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Detection of Two Pathogens of High Importance to The National Poultry Improvement Plan: Salmonella spp. and Mycoplasma spp.

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Detection of Two Pathogens of High Importance to the National Poultry Improvement Plan: *Salmonella spp.* and *Mycoplasma spp.*

DETECTION OF TWO PATHOGENS OF HIGH IMPORTANCE TO THE NATIONAL POULTRY IMPROVEMENT PLAN: *SALMONELLA SPP.* AND *MYCOPLASMA SPP.*

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Abstract

In 1935, the National Poultry Improvement Plan (NPIP) was created to control *Salmonella gallinarum* and *Salmonella pullorum*. These two pathogens were devastating economically for poultry producers. Through cooperative efforts using vaccination and strict biosecurity, these two pathogens were eradicated from the United States. Currently, the NPIP program is targeting two other poultry pathogens, *Salmonella enterica serovar* Enteritidis and *Mycoplasma.* In the broiler industry it targets 2 specific *Mycoplasma* species (*synoviae, gallisepticum*). Vaccinations for these bacteria are available, but are not fully effective at controlling all strains and serovars. Thus, constant monitoring systems and strict biosecurity measures are necessary in order to limit contamination of poultry flocks. For primary breeders, it is not permissible to sell breeding stock that is infected with *Salmonella* Enteritidis, *Mycoplasma galisepticum* or *Mycoplasma synoviae.* The work reported here is intended to benefit both producers and consumers. Considering the economic impact that these pathogens can have on producers, optimal and rapid detection methods are extremely useful. Furthermore, elimination of *Salmonella* at the farm level can be facilitated also using rapid diagnostic assays. The assays described in this work can potentially reduce the economic and health burden of *Salmonella* by aiding producers to reduce the risk of *Salmonella* infection in poultry flocks.

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Table of Contents

Chapter One:

LITERATURE REVIEW…… 1
References…….. 29
Tables and figures…… 38

Chapter Two:

OPTIMIZED CULTURING AND NUCLEIC ACID BASED METHODS FOR THE DETECTION OF *SALMONELLA ENTERICA* IN POULTRY ENVIRONMENTS…… 41
Abstract…… 42
Introduction……. 43
Materials and methods…… 44
Results……. 48
Discussion…… 50
References…… 53
Tables and figures…… 55

Chapter Three:

DETECTION OF *SALMONELLA SPP*. SURVIVAL AND VIRULENCE IN POULTRY FEED BY TARGETING THE *HILA* GENE…… 62
Abstract…… 63
Introduction…….. 64
Materials and methods……. 65
Results……. 70
Discussion……... 72
References……... 75
Tables and figures…… 78

Chapter Four:

DEVELOPMENT OF A REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR THE SIMULTANEOUS DETECTION OF *MYCOPLASMA GALLISEPTICUM* AND *MYCOPLASMA SYNOVIAE* UNDER INDUSTRY CONDITIONS……... 81
Abstract…… 82
Introduction……. 83
Materials and methods…… 84
Results……. 89
Discussion…… 90
References…… 94
Tables and figures…… 96

CONCLUSIONS…… 99
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Chapter One

Literature Review

1. Introduction

In 1935, the National Poultry Improvement Plan (NPIP) was created to control *Salmonella gallinarum* and *Salmonella pullorum*. These two pathogens were devastating economically for poultry producers. Through cooperative efforts using vaccination and strict biosecurity, these two pathogens were eradicated from the United States. Currently, the NPIP program is targeting two other poultry pathogens, *Salmonella enterica* serovar Enteritidis and *Mycoplasma.* Vaccinations for these bacteria are available, but are not fully effective at controlling all strains and serovars. Thus, constant monitoring systems and strict biosecurity measures are necessary in order to limit contamination of poultry flocks. For primary breeders, it is not permissible to sell breeding stock that is infected with *Salmonella* Enteritidis, *Mycoplasma galisepticum* or *Mycoplasma synoviae.* The work reported here is intended to benefit both producers and consumers. Considering the economic impact that these pathogens can have on producers, optimal and rapid detection methods are extremely useful. Furthermore, elimination of *Salmonella* at the farm level can be facilitated also using rapid diagnostic assays.

2. *Salmonella*

Salmonella is the leading cause of foodborne illness in the United States. In 29% of *Salmonella* infections, or approximately 406,000 cases annually, poultry has been identified as the primary source of infection (1,2). The costs associated with non-typhoidal *Salmonella* infections are estimated at nearly $2.4 billion dollars annually, which includes cost due to loss of productivity and medical treatment costs. A poultry producer suffers losses due to *Salmonella* infection of the flock including loss of birds and production time. These losses in the United States per year have

been calculated to be approximately $64 million - $114 million, but these calculated losses do not take into account the loss of eggs and other consumable poultry products.

The use of antibiotics to control *Salmonella* in poultry is not an option and alternatives to antibiotics for control of bacteria in poultry including bacteriophage and probiotics have yet to be completely successful. If one bird in a flock becomes infected with *Salmonella*, the infection can spread rapidly and the entire flock can become infected within 2 to 10 days. Since *Salmonella* may remain in the environment between flocks, control of infection initially can help reduce and eliminate environmental contamination. Hence, constant monitoring and rapid detection are needed to prevent *Salmonella* infection in poultry flocks.

Salmonella may be introduced to a flock by multiple environmental sources, but poultry feed is suspected to be a leading source. Detecting *Salmonella* in feed can be challenging due to the uneven distribution of contamination and the low levels of the bacteria may not be recovered using traditional culturing techniques. Numerous detection methodologies have been examined over the years for quantifying *Salmonella* in feeds and some have proven to be more effective for *Salmonella* isolation and detection in a variety of feeds. However, given the potential need for increased detection sensitivity, molecular detection technologies may be the best candidate for developing rapid sensitive methods for identifying small numbers of *Salmonella* in the background of large volumes of feed. The primary difficulty with routine application of molecular assays is the problem of extracting and recovering representative samples from feeds for molecular analyses. Molecular techniques may also be hindered due to chemicals present in feed samples that can inhibit PCR reactions. This review will discuss the processing of feeds and potential points in the process that may introduce *Salmonella* contamination to the feed. Detection methods currently used and the need for advances in these methods also will be

discussed. Bead-based DNA arrays for simultaneous detection of multiple *Salmonella* serotypes offer new possibilities for rapid detection and these innovations are presented. Finally, implementation of rapid detection for optimizing control methods to prevent and remove any *Salmonella* contamination of feeds will be considered.

3. *Salmonella* in Broiler Breeders

It has long been recognized that breeding stock of poultry play a crucial role in controlling the dissemination of *Salmonella* infection and contamination (3,4). Young chicks in the hatchery are more susceptible to infection with *Salmonella* due to an absence of protective gut microflora. For this reason, 1-day old chicks can be colonized with as few as 5 cells of *Salmonella*, but colonization of 2 week old birds which have protective microflora is inconsistent and requires higher doses (5). Furthermore, the susceptibility of these young chicks results in rapid horizontal transmission (6). Surveys and estimates of *Salmonella* positive chicks leaving the hatchery range from 4.8 to 9% (7,8). The dissemination of *Salmonella* from broiler breeder flocks to farm environments and possible routes of persistence are diagramed in Figure 1.

Infected chicks from a hatchery that are placed on a grow-out farm can act as sources of infection and contamination to the farm environment (9). *Salmonella* has been demonstrated to persist in farm environments for 1 year with or without poultry being present (10). Furthermore, total disinfection of grow-out farms may be impossible to achieve due to cleaning difficulties and environmental reservoirs such as mice and wild birds (11).

Salmonella contamination on the broiler grow-out farms is complex and can come from multiple sources in the environment such as feed, feed ingredients, water, litter and from breeding stock

(6,7,12-16). However, it is more difficult to determine the sources of *Salmonella* to primary broiler breeder flocks. Primary breeding flocks are substantially more valuable than other poultry stock and, therefore, the hatchery design is usually state-of-the-art with a one-way movement of clean to dirty flow design to reduce contamination. Incidences are typically lower in broiler breeder hatcheries (17). These breeder flocks are much smaller and hatching eggs are gathered more frequently and disinfected shortly after being gathered (4).

Feed has been implicated as an important source of *Salmonella* to poultry (18,19). Hinton (20) demonstrated that *Salmonella* infection could become established in day old chicks fed 0.1 to 0.3 cells of *Salmonella* per gram of feed. Modern culturing techniques require enrichment in order to detect such a low number of cells and molecular techniques are not sensitive enough to detect such low numbers. For this reason, some *Salmonella* contamination of feed may pass undetected. Sources of *Salmonella* and the processing of feed will be discussed in the next section.

4. *Salmonella* and Feed

It has been suggested that occurrence of *Salmonella* contamination in feeds produced in feed mills may be due to transfer of *Salmonella* from birds, rodents or other pests (21). In addition, contamination of feed mill ingredient intake pits and outloading gantries for finished feed products by wild-bird droppings containing *Salmonella* has been described (22). Pelleted and mash poultry feeds have long been recognized as vectors for *Salmonella* contamination in poultry production systems with ingredients of animal origin having the highest frequencies of contamination (23,24). However, ingredients of vegetable origin also have been reported to

harbor the organism (18). Since animal feed is the first portion of the farm to fork continuum for food safety, it represents a critical point for intervention and control of *Salmonella.*

4.1. Sources of Salmonella to Feed

Morris *et al*. (25) found that of all the samples taken from a commercial broiler operation, feed samples were most frequently contaminated with *Salmonella* . Human outbreaks of salmonellosis have been traced back to feed for decades. In 1958, an outbreak of infection of *S.* Hadar in Israel was linked to the consumption of chicken liver and was eventually traced back to bone meal fed to the chickens (26). Frozen chickens from a packing plant in Cheshire, England were implicated in a large outbreak in 1968 of infection with *S.* Virchow (27). The investigation showed that the hatchery and some rearing farms that supplied the packing plant contained chickens colonized with *S.* Virchow. In this investigation, the same serotype of *Salmonella* was isolated from feed fed to the chickens (28). Chickens served in a restaurant in Arkansas caused an outbreak of *S.* Agona. The chickens were traced to a farm in Mississippi that fed the chickens with feed containing Peruvian fish meal found to be contaminated with *S.* Agona (29) The fish meal was found to be the ultimate source for a number of *S.* Agona infections in the United States, the United Kingdom, Israel, and the Netherlands.

Several more recent investigations using sophisticated genotyping methods have confirmed that *Salmonella* in feed can be a primary source of contamination. In a study by Shirota *et al*. (30), *S.* Enteritidis strains obtained from feed samples and egg contents taken from a layer farm showed pulsed-field gel electrophoresis (PFGE) patterns that were genetically related. Futhermore, the isolates belonged to a single phage type which suggested that the contamination of the farms was

linked to the occurrence of *Salmonella* Enteritidis in feed. Using PFGE, Wasyl *et al*. (31) found *Salmonella* isolates with identical pulse-types isolated from feed and poultry. Bucher *et al*. (32) also used PFGE along with serotyping, phage typing and antimicrobial resistance typing and concluded that *Salmonella* strains isolated from broiler feed were indistinguishable from strains isolated in packaged raw, frozen chicken nuggets and strips.

4.2. Processing of Feed

Feed is typically comprised of corn, soybeans, oats, alfalfa, calcium and a vitamin mixture (33). This composition may vary depending on the manufacturer and the type of poultry being fed. For example, laying hens require higher concentrations of calcium for egg shell production. To produce the feed, ingredients are mixed and steam processed. After processing, the feed is cooled by passing through a cooling air unit or, prior to cooling, pelleted into a cylinder-like shape. Himathongkham *et al*. (34) demonstrated that feed moisture and conditioning time were two factors that play a crucial role in the lethality of the pelleting process for bacteria. Most studies agree that the pelleting process is more effective at reducing *Salmonella* contamination. Cox *et al*. (24) reported that 92% of mash feed samples were positive for *Salmonella* but no pelleted samples were positive. However, Veldman *et al*. (18) found 21% of mash feeds and 1.4 % of pelleted feeds were positive for *Salmonella*. Similarly, Jones *et al*. (19) found that 8.8% of mash feed samples and 4.2% of pelleted feed samples were contaminated with *Salmonella*.

If *Salmonella* is destroyed during the heat treatments, the possibility of re-contamination still exists. Raw feed ingredients can serve as a source of contamination to the plant environment and ultimately to the final feed product. Veldman *et al* (18) sampled raw feed ingredients and found

130 samples of fish meal (31%), 83 samples of meat and bone meal (4%), 58 samples of tapioca (2%) and 15 samples of maize grits (27%) were positive for *Salmonella*. The data presented by Jones *et al*. (19) indicated that dust within feed manufacturing facilities could serve as a major source of contamination to the final product. The authors suggested that mechanical vibrations and air currents around the pellet mill might have resulted in dust particles being dislodged and landing on the final pelleted feed. Davies and Wray (22) showed that the cooling unit was colonized by *Salmonella* which might serve as an airborne source of contamination to the final feed product. In addition, there is the possibility of feed being contaminated during transportation and / or storage (35). With the possibilities of post-process contamination, detection methods are critical for preventing flock contamination. The next section will address current detection methods available and possibilities for future developments.

5. Detection of *Salmonella*

5.1. General Concepts

Detection of *Salmonella* in feed can be challenging due to low number of cells present in a large volume of feed. Riley (36) estimated a contamination rate of feed passing through a contaminated cooler would pick up 1 *Salmonella* organism per 10 to 100 tons if the facility was not receiving feed ingredient loads that were contaminated. At such a level of contamination, the challenge becomes designing both a sampling program and a method of detection that can detect 1 cell in 10 tons of feed.

Complications associated with isolating *Salmonella* from feed not only has been suggested to stem from the non-uniform distribution of the organism within the samples, but also from the effect of stress on the organisms from processing treatments used in feed mills (18,19,37). In addition, the treatment of feed with organic acids or formaldehyde can interfere with detection methods and give a false negative result (38).

Numerous detection methods have been developed for *Salmonella* such as culturing, immunological methods and nucleic acid based methods (40). Typically, the method is chosen based on the application of the user. For example, if the desire is to not only detect but also to characterize *Salmonella* , the isolate will need to be recovered by culturing for further genotyping, antibiotic resistance typing, serotyping or other characterizations. However, if only presence or absence is necessary then nucleic acid detection assays are sufficient. Each method has advantages and disadvantages and every method has some limitations. The following sections will discuss the methods available and describe shortcomings and benefits for using a particular assay when applied to poultry breeder feeds.

5.2. Culturing

Traditional microbiological culture methods for the detection of *Salmonella* in feeds include selective enrichment and selective and / or differential plating. Culturing methods for the detection of *Salmonella* have been reviewed (39,40). In general, plate agar media contains a pH indicator and lactose to differentiate *Salmonella* (a fermentor) from non-fermenting bacteria. Since most *Salmonellae* are hydrogen sulfide producers, ferric citrate can be added which results

in *Samonella* colonies turning black. Other selective agents may be added such as antibiotics like novobicin which permit the growth of *Salmonella* while inhibiting competing microorganisms. Chromogenic media work by using enzyme substrates that release colored dyes after hydrolysis, resulting in *Salmonella* colonies being colored and easily differentiated from other flora. This type of agar has an increased specificity over conventional media. However, some reports have shown that conventional media are less inhibitory and, therefore, more sensitive because stressed or injured microorganisms can be recovered (41,42). Regardless of the plating media used, culturing methods are often considered as the "gold standards" but are time consuming in that they require days for results. Given that infection of an entire flock can occur in as few as three days (43), a method of detection that is more rapid than culturing is needed to implement control measures and control any further spread of infection.

5.3. Serology and Immunoassays

The genus of *Salmonella* consists of only two species, *S. enterica* and *S. bongori* but over 2,500 serotypes (44). There are 47 possible *O*-antigens (lipopolysaccharide of cell wall) and 60 possible *H*-antigens (flagellum) with each serovar having its own unique combination of *O*- and *H*-antigens (45). The name of each serovar was given based on the syndrome displayed, host specificity or geographical location (45).

Each serovar of *Salmonella* may vary widely in characteristics including severity of disease, virulence properties, ability to colonize chickens and survival in the environment outside the host (46-49). For this reason, identification beyond the species level is necessary. Furthermore,

serovar information is used by local and state health departments and CDC to monitor local, regional, and national trends of salmonellosis.

Serological based assays (immunoassays) such as ELISA have been widely used for *Salmonella* detection because they enable sensitive and specific detection (50). Immunological methods for the detection of *Salmonella* in animal feeds have been reviewed (40). Immunoassays offer the ability to detect and distinguish serovars of *Salmonella.* However, immunoassays are difficult to incorporate into an array format for multiple targets because high level of cross-reactivity between antibodies limits the number of targets that can be detected on the same array. Use of immunomagnetic beads (IMBs) in detection assays has been reported, and it shows great potential in *Salmonella* detection from complex matrices or environmental samples (51,52). In IMB applications, however the number of target analytes detected in a single assay has been restricted to one or two due to high cross-reactivity and limited availability of commercial IMBs or antibodies for specific target organisms.

5.4. Nucleic Acid Detection

Polymerase Chain Reaction (PCR) has been gaining popularity as a tool in microbiological diagnosis due to the efficient, rapid and sensitive methods of detection. The methodology of PCR for the detection of foodborne pathogens has been reviewed previously (53). Several variations of standard PCR, such as multiplex PCR and real-time PCR, have recently been employed for *Salmonella* detection, and these methods have provided high sensitivity with some assays being able to detect as few as 30 cells per sample (54). The important criteria in the development of a nucleic acid based detection assay for *Salmonella* is the ability to detect all the diverse serotypes

of the organism and PCR has been employed to replace conventional serotyping methods (55). PCR-based serotypings depend on specific genes many of which are virulence genes, and have provided high specificity (56,57). However there is a limitation on the number of target *Salmonella* serovars which can be detected in single PCR reaction. Even in multiplex PCR, it is difficult to incorporate more than five to six primer sets (correlating to five or six serovars) in one reaction due to cross-reactivity. Considering that there are at least 12 serotypes of *Salmonella* commonly associated with poultry (58), there is a clear need for an assay able to simultaneously detect multiple *Salmonella* serovars with minimal cross-reactivity.

5.5. Biosensors

Biosensors are being developed because they offer rapid and sensitive unconventional detection methods. Biosensors generally contain two components, a biological material (nucleic acid or antibody) closely associated with a transducing system. The transducer emits a signal when the target is captured that can be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical. Biosensors differ from conventional detection methods because they are self-contained single units that have both the detection and reporting components.

Optical biosensors which utilize a fluorescent signal are typically the most common type of sensor (59). However, biosensors using transducers other than optics have been developed for the specific detection of *Salmonella*. Olsen *et al*. (60) utilized bacteriophage specific for *Salmonella* Typhimurium. In this biosensor, the capture of bacteria by bacteriophage that was adsorbed to a piezoelectric transducer resulted in a resonance frequency change measured with a Maxtek acoustical wave device. Su *et al*. (61) used an antibody bound to a gold coated quartz

crystal surface with a gold electrode as a biosensor. After capturing *Salmonella*, changes in high-frequency impedance were directly correlated to the number of captured *Salmonella* cells. Pathirana *et al*. (62) and Kim *et al*. (63) also used a similar impedance analysis to create biosensors for the detection of *Salmonella* Typhimurium.

DNA microarrays are a type of optical biosensors that detect hybridization of DNA sequences between bound probes having known sequences to fluorescently labeled DNA from an analyte. If hybridization is successful, a fluorescence signal is emitted upon excitation with a laser and the intensity of the signal can be used for quantification and identification of the analyte. Planar DNA microarrays allow thousands of specific DNA sequences to be screened simultaneously on a small single glass slide. Using DNA microarrays, multiple *Salmonella* serovars can be concurrently detected and the presence of virulence genes and antibiotic resistance genes can also be identified at the same time (64,65). While planar microarrays offer the great potential for a rapid and sensitive detection of multiple pathogens (66,67) high fabrication cost and requirement for expensive equipments have been limiting their wide application in routine applications (68).

Bead-based microarrays, as an alternative to planar microarrays, have been developed to perform multiplexed detection assays (68,69,70). In bead-based arrays, microspheres are employed as solid support for the capture molecules (e.g. antibodies, oligonucleotide probes), instead of glass slides conventionally used in planar microarrays (Figure 2).

The individual microspheres are color-coded by distinct fluorescent dyes. In each DNA microarray, the oligonucleotide probe is immobilized to the surface of a distinct type of microspheres which are chemically functionalized. Different bead sets are then pooled to create a library, and hybridization is performed in a single vial or single well in a 96-well microplate

containing the library of all bead types. After hybridization, presence of targets can be detected with a two-laser flow cytometer, where one laser interrogates the encoding dyes of beads to determine the probe identity and the other laser determines the presence of targets in the sample by reading the second fluorescent signal from hybridized targets.

Bead-based DNA arrays have several advantages over planar microarrays; (1) they can accommodate standard 96-well sample preparation systems; (2) since probes are coupled to distinct microspheres, each hybridization reaction can be analyzed; (3) if an additional target has to be included into the assay, a new type of probe-loaded bead can simply be added to the array unlike planar microarrays which require the fabrication of new arrays to add a target (68,69). Bead-based arrays coupled with flow cytometry technology have been successfully applied for the simultaneous detection of multiple bacterial pathogens (71); however this study was done with pure culture of target pathogens. Bead-based arrays have never been employed for detection of pathogens in more complex matrices such as feed or environmental samples. Bead-based arrays have been more commonly used in clinical applications such as simultaneous quantification of cytokines or autoantibodies from biological samples (72-75). Bead-based arrays have the great potential for rapid and sensitive identification of *Salmonella* from feed. The criteria for optimal bead based array design are listed in Table 1.

An initial step for rapid microarray development involves the selection of target genes and design of probes and primers that detect and characterize *Salmonella* spp. which are commonly found in poultry breeder feeds. However, effective sample preparation methods to minimize the effect of environmental factors are usually required to retrieve *Salmonella* from feed matrices. Immunomagnetic separation using anti-*Salmonella* magnetic beads can be employed as a standard method to separate *Salmonella* from feed matrices (76). Cultural pre-enrichment also

can be utilized to optimize sample preparation to alleviate any inhibitory effect from feed matrices while keeping the total assay time short.

Both optical and electrochemical biosensors offer advantages, but also come with disadvantages. Optical techniques have been demonstrated to provide better sensitivity than electrochemical ones (59). Electrochemical techniques offer simplicity over optical detection methods. However, optical techniques offer the ability to capture and detect many targets and for this reason are usually more costly. Some biosensors are sensitive but they still are not capable of the same detection levels as traditional techniques.

6. Future prospects for *Salmonella* detection

Sweden implemented a Hazard Analysis and Critical Control Point (HACCP) program for animal feed in 1991 and since then a decline in the annual incidence of domestically acquired human salmonellosis has been observed, with a drop from 14 cases per 100,000 population in 1991 to 8 cases per 100,000 population in 2000 (77). Under the Swedish HACCP program, approximately 7,000 samples from feed mills are analyzed annually, of which 40% are obtained before heat treatment. Detection of any positive samples generates more sampling and corrective actions are taken. Sweden has an integrated surveillance of feed, animals, food, and humans which allows investigators to track trends and monitor the impact of interventions and has virtually eliminated *S. enterica* from domestically produced animal feed and red and white meat (77,78). Jones *et al*., (7) also underlined the need for a comprehensive approach in the control of *Salmonella* contamination in broiler production and processing systems. As a follow up to this point, Jones and Ricke (79) outlined a specific Hazard Analysis of Critical Control Points

approach for the control of *Salmonella* in feeds. Presently, a farm or feed mill in the U.S. may adopt several good manufacturing practices (GMPs) to reduce feed recontamination. Feed bins, feed pans, cross augers, hoppers, silos and transport trucks and silos could be regularly cleaned and painted with ceramic paint to prevent the buildup of caked feed that may be contaminated with pathogenic molds, bacteria, or mycotoxins (80,81). Systems have been in development which may disinfect truck tires while the truck is still moving, reducing soil contamination between the farm and feed mill (82). Dust in feed mills may be sampled for airborne *Salmonella* spp., a general indication of *Salmonella* spp. presence in the environment that may reduce the problem of sample size (83). Monitoring *Salmonella* spp. either in feed mixtures or feed ingredients will probably require some sort of direct detection of *Salmonella* spp. Improvements in detection methods that are more sensitive and rapid are needed to control *Salmonella* from a top down approach. Because only a few cells of *Salmonella* can infect a chick, sensitivity of an assay is crucial. Furthermore, given the rapid transmission of *Salmonella* within a flock, an assay that could be performed in less than 24 hours would give producers time to implement corrective actions and control transmission.

7. *Mycoplasma*

7.1 Description of Mycoplasma

Mycoplasma spp. are prokaryotes that lack a cell wall but have a cell membrane. The name Mollicutes is derived from the latin for soft skin. *Mycoplasma* is taxonomically placed in the class Mollicutes, order Mycoplasmatales, and family Mycoplasmataceae. The genus

Mycoplasma is distinguished from *Ureaplasma* in the family by an inability to hydrolyze urea. Members of the genus have a small genome (580 to 1350 Kb) and relatively low G+C % content (84). The small genome size is clearly reflected by the reduced metabolic capabilities of mycoplasmas. Mycoplasmas lack pathways for cell wall production and biosynthesis of purines and also lack a functional tricarboxylic acid (TCA) cycle and a cytochromemediated electron transport–chain system. These organisms must obtain many of the necessary nutrients from the environment needed to multiply and survive. For this reason, mycoplasmas are obligate parasites. This characteristic is also reflected in the ideal culturing temperature (37^0C) the same body temperature as that of humans and many animals.

Mycoplasma are the smallest self-replicating organisms. In the 1950's Klinenberger-Nobel discovered a loss of the cell wall in bacteria that were treated with antibiotics or lysozyme and noticed that the organisms were still able to divide even with a lack of cell wall. These organisms were called L forms. Currently, there are more than 120 named *Mycoplasma* species (http://www.ncbi.nlm.nih.gov/).

7.2 Mechanisms of Pathogenesis

Mycoplasma has a variety of animal hosts including humans and are capable of producing disease in many of these hosts. Of the 120 named species, 20 infect poultry with *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) being most commonly isolated from chickens (85). Mycoplasmas typically cause respiratory diseases in their host and in chickens the disease is characterized by coughing, nasal discharge, and air sac lesions, but in some infections no clinical symptoms appear (86).

Although mycoplasmas are typically isolated from the respiratory tract, they have also been isolated from the reproductive organs, brain and eyes of poultry. Once infected, mycoplasmas must adhere to the surfaces of epithelial cells for successful colonization. The molecular mechanisms of pathogenesis have been investigated and along with whole genome sequencing, much of the disease process has been described (84).

Research into the molecular mechanisms of *M. gallisepticum* attachment and subsequent virulence has identified a specialized terminal organelle, or bleb-like structure, that serves as an attachment tip (84). Other potential adhesion structures include surface proteins containing highly reiterated domains. These proteins are members of large gene families, and individual members often undergo high-frequency phase variation which is thought to promote evasion of the host immune system (87).

Current theory argues that mycoplasmas remain attached to the surface of epithelial cells and invasion is either not likely or does not occur significantly (88). During attachment, damage to host tissues takes place releasing nutrients that can be utilized. Mycoplasmas primarily infect the respiratory tract causing damage to the ciliated epithelial cells lining the trachea. Ciliostasis results and mucus is not moved upwards out of the trachea which also prevents the organism from being removed.

During attachment of mycoplasmas to the surface of host cells, interference with membrane receptors or altered transport mechanisms of the host cell can occur. Although no known toxins have been described, mycoplasmas can produce metabolites and enzymes that are toxic to the epithelial cells. Mycoplasmas may also hydrolyze phospholipids utilizing phospholipases which compromises the host cell membrane. In addition, the host cell membrane is also vulnerable to peroxide and superoxide radicals (89).

7.3 Costs for poultry production

MG and MS are the most common poultry pathogens and can impact breeder, broiler, and egg laying production. For layer operations, reductions in egg production are estimated at $140 million annually (90). In broilers, a reduced feed conversion efficiency, depressed growth rate, and condemnation of carcasses can be economically devastating. Losses as high as $750,000 have been reported from a single outbreak of MG (91).

Economic burdens of MG and MS also include the cost of monitoring and detection. Culturing is a time consuming and lengthy process requiring multiple types of media and regular man hours. Serology is more rapid, but costs are also high for this method. Molecular based approaches are less costly, however, the initial investment in equipment can be expensive. For some producers, especially breeders, the choice may be to utilize a combination of all three for confirmation and assured detection. This approach can be quite costly, but may be worth the investment considering the cost of a loss of a breeder flock.

8. Detection methods for *Mycoplasma*

Antibiotics can be used to treat poultry for a *Mycoplasma* infection, but may not be fully effective at clearing the infection (92,93). In most instances, it is necessary to eradicate the entire flock. Because mycoplasmal infection may not result in outward symptoms, a stringent biosecurity and biosurveillance practice which can facilitate early intervention strategies are necessary to control mycoplasmal infections. Currently, methods for detecting *Mycoplasma*

infection that are typically used include culture, serology or molecular assays. Traditional culturing is not commonly utilized because the method is time consuming, they are slow growing, and some fastidious strains may not be detected (94). Serology is much faster than culturing, but disadvantages of serology include non-specific reactions and cross-reactions between species, mis-interpretations due to recent vaccination, and cost (95). Furthermore, antibodies to MG and MS may not be detected until 1 to 3 weeks post-infection (85). The following sections will describe these three techniques for detection and give advantages and disadvantages for each method.

8.1 Culturing

As discussed in the earlier sections, culturing of *Mycoplasma* can be quite difficult due to the fastidious nature of the organism. Typically tissue samples are acquired from the respiratory tract such as the lungs, air sacs, or trachea. If whole organs such as lungs are utilized, a lavage can be performed with phosphate buffer saline (PBS). Inhibitors may be released from the host tissues during isolation if tissue is ground, but this problem can be overcome with the addition of chemicals or by diluting the sample.

The samples are typically enriched in a broth medium with a meat-infusion base prior to plating. MG and MS require cholesterol and other fatty acids as a nutrient source. Supplemental antibiotics that target and inhibit competing organisms are also added, these antibiotics usually target the cell wall and since *Mycoplasma* lacks cell wall they do not inhibit our target *Mycoplasma*. Frey et al. (94) developed a culture medium that is widely used in the United States of America (USA) and other countries for isolation of MG and MS. Nicotinamide adenine

dinucleotide (NAD) and cysteine are added for the isolation of MS, but it may be omitted for the cultivation of MG. A 0.6 to 0.8% agar is typically utilized with a neutral pH (7.4 to 7.6) and plates incubated at 37C in a moist environment.

Colonies display a fried egg shape on agar. For confirmation, commercially available antibodies with fluorescent tags can be used as well as growth tests utilizing antiserum. Preservation of cultures is similar to preservation of most bacteria. Freezing at lower temperatures will preserve the cultures for an extended period of time and adding a cryoprotecting reagent can also extend the life of the culture.

Culturing is considered the gold standard. Isolating mycoplasma can be very useful for further diagnostic and future epidemiological studies. Pure cultures can be characterized phenotypically and genotypically which makes culturing advantageous over serology and molecular based detection techniques. However, due to the sensitive nature of this organism, culturing can be labor intensive and unsuccessful. For example, Jarquin et al. (96) compared isolation techniques and found culturing produced the greatest number of false negatives as compared to serology and molecular detection techniques. The authors suggested that the time gap from sample collection to processing may have resulted in loss of cultures. In addition, the study pointed out that freezing the tissue samples may have also affected culture recovery.

8.2 Serology

Serological based assays utilized in poultry are aimed at detecting any antibodies produced by the host in response to *Mycoplasma* infection. Blood is collected from the birds and the collected sample is allowed to separate. The serum then can be used in an antibody based assay. Assays

are usually in one of three formats: plate agglutination, hemagglutination inhibition (HI), or ELISA (enzyme labeled immunosorbent assay). Plate agglutination detects IgM, while HI and ELISA detect IgG.

Plate agglutination is a very simple assay in which serum is mixed with MG and MS antigens on a glass slide and positive results can be rapidly visualized by clumping due to the antibody binding with the antigens. Plate agglutination detects IgM antibodies which are pentamers and, thus, bind well to antigens. The general term agglutinin is used to describe antibodies that agglutinate antigens. When the antigen is an erythrocyte the term hemagglutination is used. For *mycoplasma* specifically, the plate agglutination is test where serum is mixed with MG and MS antigens.

Because hemagglutination inhibition (HI) detects IgG, thus infection cannot be detected as early with HI when compared to plate agglutination. The assay is performed in a microtiter plate composed of 96 wells. HI positive agglutination results do not agglutinate (inhibition of agglutation of erythrocytes) due to the antibody-antigen binding. A microtiter plate can be used where each well has a varying concentration of antibody. In this way, it is possible to quantify the level of antibodies present in the serum sample.

Plate agglutination and HI assays can both exhibit false positives, with plate agglutination more prone to false postives. Several factors can lead to false positives but the primary contributor is vaccination. Vaccination to MG and MS stimulates the production of antibodies (that can cross react with MG and MS antigens) that can circulate for 2 to 5 weeks. Contaminated serum, frozen and thawed serum, and cross-reactions to other antibodies can also cause false positives. False positive reactions can be reduced by heating serum to 56°C for 30 minutes or by diluting serum (97).

ELISA is the third type of antibody-antigen detection-based assay. In this assay, antibodies or antigens are bound to the wells of a microtiter plate. The wells are then filled with diluted serum and given time for the binding reaction to occur. The wells are washed and a secondary antibody or antigen that is tagged with a color is then added. Positive reactions can be visualized by noting a color change.

HI and ELISA are typically used as confirmational assays for the simple plate agglutination assay. HI and ELISA are comparatively more labor intensive and thus, not utilized as a primary method. These two methods also take more time than simple plate agglutination.

8.3 Molecular assays

Molecular based techniques have become increasingly popular. Polymerase chain reaction (PCR) assays which target and detect specific nucleic acid sequences, can give results in less than 24 hrs. Real-time PCR also detects specific nucleic acid sequences but utilizes a fluorescent based system so the amplification of the target can be monitored during the reaction. Real-Time PCR has additional advantages over traditional PCR including: 1) real time is more rapid and can be accomplished in as little as 40 minutes; 2) no post-amplification processes are required which decreases total detection time, cost in terms of materials, and hazardous waste; 3) are more sensitive - some real time assays can detect as few as 10 template copies per 5 μl sample; 4) questionable results can be confirmed using melting curves.

Most PCR based methods require the sample be suspended in a non-nutrient medium. Specific to poultry, cleft palentine swabs are usually performed and the swab is then suspended in nuclease free water to release the sample from the swab. Samples are subsequently heated to boiling

which lyses the cells and releases the nucleic acids. Centrifugation of this preparation collects debris in the pellet while target nucleic acids remain in the supernatant.

There are several molecular assays available for detection of MG and MS. Jarquin et al. (96) and Hess (98) utilized primers that targeted the 16S ribosomal subunit. Hong et al. (99) designed their primer set to target the *vhlA* gene. The *vhlA* gene is typically utilized for genotyping and differentiating strains. Thus, the authors were able to detect and sequence the PCR product which facilitated epidemiological tracking efforts. Ramirez et al. (100) targeted the interspacer region (ISR) between the 16S and 23S rRNA genes to detect and distinguish MS from 22 other poultry *Mycoplasmas*. Raviv et al. (101) used the same approach for MG. All of these different primer sets have not been compared therefore it is not known whether one primer set is more accurate or sensitive than another.

9. Intervention methods for *Mycoplamsa*

As discussed earlier, intervention measures are typically not performed for infected birds. A constant monitoring program is a key to early intervention. In addition, a strict biosecurity protocol is also very helpful for preventing infections with MG and MS. Entire flocks can become infected in 2 to 10 days (86) and given that antibiotics may take 3 days to be effective, the infection can be difficult to control once it has begun. Thus, the course of action is dependent on many factors including on the type of birds that are being produced. The next section will discuss three types of production operations and how MG and MS a controlled in these operations.

9.1 Breeders

Primary breeder operations are by far the most expensive of all three types of operations. In these systems, genetic lines of birds are well established and specific traits are maintained through genetic selection. Operations typically utilize farms for production however the farms are state of the art and kept extremely clean. The cost of one bird can be as great as $5,000 and thus much time and effort is invested into maintaining a healthy population.

Primary breeders operate under the National Poultry Improvement Plan (102). The NPIP was formed in 1935 to target *Salmonella gallinarum* and *S. pullorum*. At this time, these bacteria were economically devastating to producers. Through cooperative vaccination and biosecurity, *S. gallinarum* and *S. pullorum* were eradicated from the U.S. Currently, MG and MS are a main focus of this program. Primary breeders operating under the NPIP must comply with the program regulations that include the vending of MG and MS free birds.

Due to the high cost of primary breeder birds, infection with MG and MS are monitored frequently. Although the cost of monitoring can be expensive, given the cost of primary breeder birds, the investment in diagnostic assays is relatively low compared the potential cost of a loss of a flock. To control infection, breeders typically destroy entire flocks if MG or MS outbreaks occur. Since vending infected birds is not allowed under the NPIP program, eradication is the only solution.

9.2 Broilers

Many large scale broiler operations house anywhere from 15,000 to 30,000 birds per house. Each bird is given approximately 1 sq. ft. (0.09 sq mt.) of space. Due to the proximity of the birds, infection spreads rapidly. In a controlled setting, Feberwee et al. (95) designed a model to measure the rate of MG transmission. In this study, all birds were housed in separate cages that were 65 cm apart (approximately 2 feet). They found transmission occurred within 14 days from infected to uninfected birds. This study primarily focused on transmission via aerosols. However, in a broiler operation there are many other factors and modes of transmission including feed and water.

For broiler operations, the course of action a producer takes is dependent on the time of infection. Broilers are typically raised for a total of 42 days prior to slaughter. Infection of young birds can lead to large losses. Younger birds have an immature immune system and cannot clear the infection. Vaccination can be done at the hatchery but vaccination is not always fully effective at preventing infection. In addition to loss of birds due to death, producers may suffer economic losses because MG and MS infections can reduce production parameters, and cause plant condemnations due to airsaculitis. Thus, even if the infection can be treated, a reduced bird size at the end of the rearing period can occur. If infection occurs late in the production cycle, a producer may not suffer any losses and no course of action may be required. Control of MG and MS in broilers has been recently reviewed (85).

9.3 Layers

Egg laying production systems can also be impacted by MG and MS. A marked reduction in egg production may result from infection with MG and MS. It has been reported that MG and MS can cause 20 to 30% reduction in egg production (103). Furthermore, eggs with pimpled shells are also associated with *Mycoplasma* infections (104). Since egg laying hens have relatively longer periods of production compared to broilers (80 weeks or more), once infected it is nearly impossible to eliminate the infection and therefore, production can be affected for the life of the flock.

Vaccination of laying hens is performed at 12 weeks of age and delivered in the drinking water (105). However, *Mycoplasma* infection can be transmitted vertically. *Mycoplasma* vertical transmission can be controlled by incubating eggs at a relatively higher temperature (46^0C). *Mycoplasmas* cannot survive this temperature, however a reduction in hatchability may result (105). Thus, like other production types rigid biosecurity and a constant monitoring system can reduce the risk of *Mycoplasma* infection.

10. Future directions for *Mycoplasma* control

Because *Mycoplasma* can be so economically devastating, control using monitoring systems and strict biosecurity are both necessary. The NPIP program has been successful in the past with eradication of other poultry significant pathogens. Whether or not MG and MS can be eradicated will be a matter of time. The program targets breeder operations and therefore uses a top down approach. By controlling MG and MS at the breeder level, it may be more effective in preventing dissemination to the production farms. One significant source of MG and MS is backyard flocks. These flocks are typically small and owned for personal use. These backyard chickens are

exposed to more wild animals which may be sources of MG and MS and biosecurity is completely absent. Thus, backyard birds can serve as a potential reservoir for the pathogens. Current research is exploring vaccines and alternatives to antibiotics. Antibiotic alternatives include treatments such as bacteriophage and recombinant vaccines. At this point, there are no treatments or preventive therapies that are 100% effective. Therefore, prevention through biosecurity and monitoring are the only options.

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Figure 1. A diagram of the possible routes of dissemination of *Salmonella* from broiler breeder flocks to farm environments and possible routes of persistence.

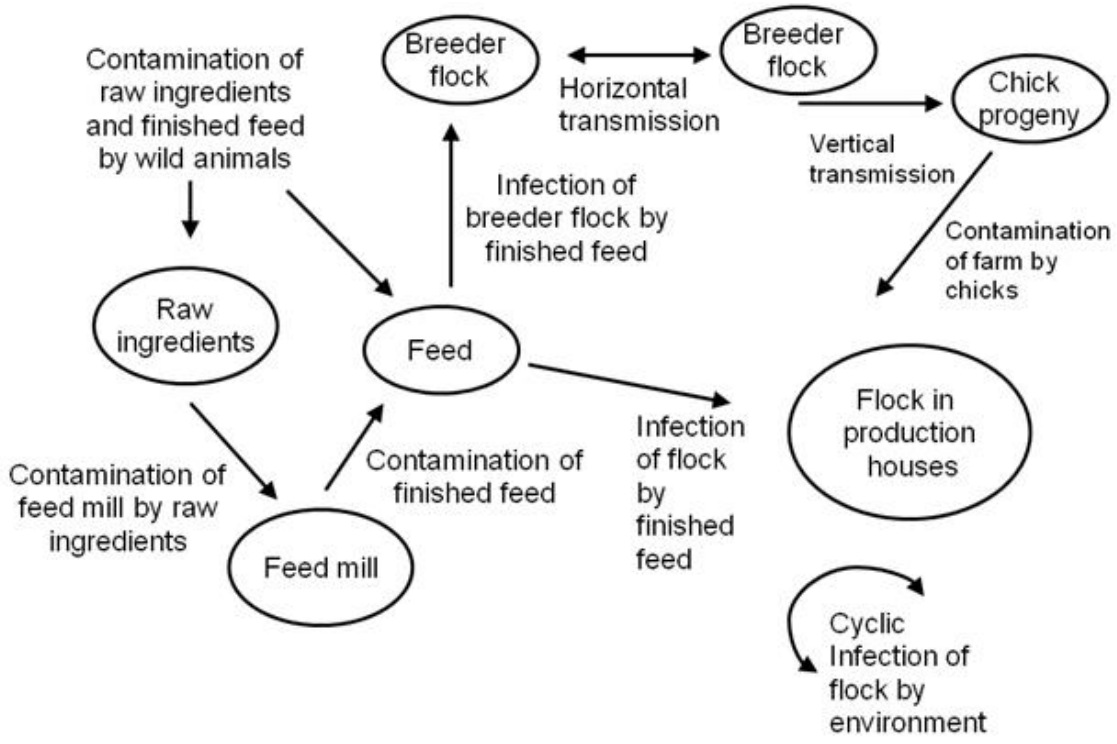

Figure 2. Illustrated description of bead based sensor array preparation, capturing of target and analysis. A, B and C denote different oligonucleotide probes (e.g. Bead A is beads functionalized with probe A). Red, green and blue colors indicate different types of encoding indicators.

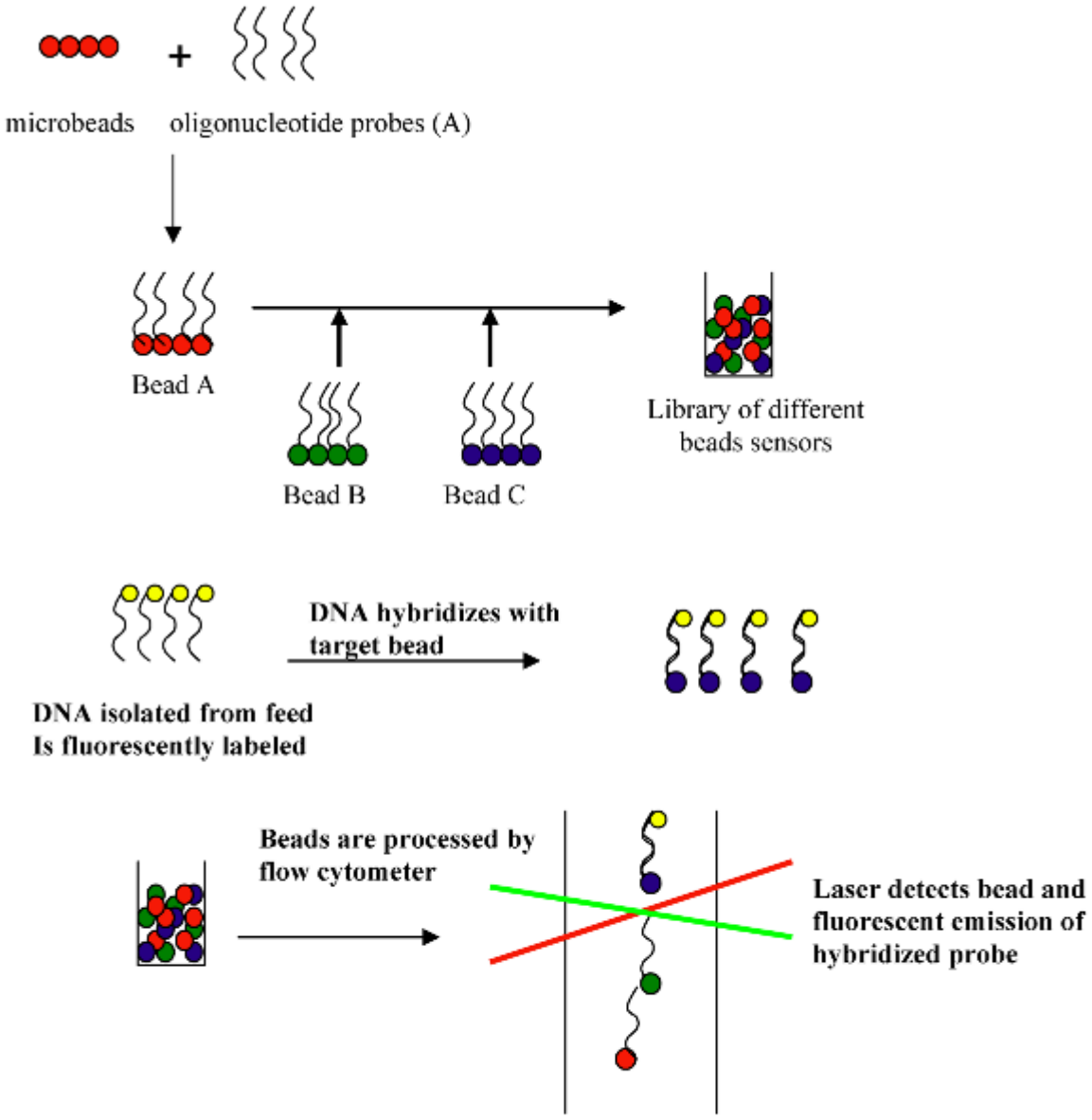

Table 1. Criteria for Optimal DNA Bead-based Microarray Detection

- Target genes and designed probe and primer sequences can detect target *Salmonella* serovars with minimal cross-reactivity.
- Bead-based DNA microarrays can simultaneously detect multiple serovars using a 96-well microplate format.
- Developed bead-based microarrays will work with both synthetic and culture samples.
- Microarrays can detect *Salmonella* pathogens in various feed and feed ingredient samples from both experimentally and naturally contaminated samples.

Chapter Two

Optimized culturing and nucleic acid based methods for the detection of *Salmonella enterica* in poultry environments.

Abstract

The main objective of this trial was to set up a series of assays following quantified inoculation of *Salmonella* samples in two initial enrichment methods to ultimately determine the most effective and early detection method for recovery of *Salmonella* in a poultry matrix. Samples were randomly split into two different containers containing either sterile 1-X solution of buffered Peptone water (BPW) + Yeast Extract, or a 1-X solution of Tetrathionate (TT) with added Iodine solution and Brilliant Green solution 0.1%. The frozen stock *Salmonella* culture was thawed and serially diluted 10-fold to inoculate 100ul of the dilution into the enriched samples. All samples were incubated at 42^0C for 24 hrs. For samples enriched in BPW, a 100 ul of the same sample was enriched in MSRV. All samples then were re-incubated under the same conditions. After enrichment, the samples were streaked onto chromogenic agar/ XLT4 bi-plates using a sterile loop and all plates were incubated under the same conditions. For samples initially enriched in TT, the same procedure was used. After initial inoculation and after each 24 hour incubation a portion of all the enriched samples was utilized in a real-time PCR assay. The results of this trial indicate that recovery of *Salmonella* in a culture based assay may be enhanced by up to 3-logs, by using the TT as the initial enrichment media compared to BPW. The incorporation of MSRV as a secondary cultural selective media after the TT yielded the best recovery of *Salmonella*. These data indicate that considerable time and expense can be saved by utilizing TT as an initial media for *Salmonella* recovery.

Introduction

Salmonella enterica is a leading cause of foodborne illness in the U.S and throughout the world (13,16). The CDC estimates that there are approximately 1.4 million cases of salmonellosis annually in the U.S. costing approximately 2.6 billion dollars in terms of loss of productivity, health care costs and premature death (12). Poultry and poultry products are a leading source of foodborne salmonellosis (3,7,10). Poultry may be colonized by *S. enterica* without causing any harm or disease to the animal. Therefore if gastrointestinal rupture occurs during processing, contamination of the raw product may occur (4).

Control of *Salmonella* on the farm is a critical point in the process of poultry production that can possibly reduce foodborne salmonellosis. One program aimed at reduction of *Salmonella* on U.S. farms is the National Poultry Improvement Program (NPIP). Broiler breeders certified and operating under this program are required to sell stock that is free of *Salmonella* Enteritidis. For these reasons, a constant and accurate monitoring program for *Salmonella* by NPIP participants is required. Monitoring assays that give the earliest detection results are ideal in order to limit vertical transfer of *Salmonella* and facilitate early corrective actions.

Culturing of *Salmonella* for detection is considered the gold standard. However, culturing can be time consuming and take as long as four days for results. Polymerase chain reaction (PCR) assays can be used as detection method with results in as few as 24 h. The main objective of these trials was to set up a series of assays following quantified inoculation of *Salmonella* into

feed and drag swab samples in two initial enrichment methods to ultimately determine the most effective and early detection and organism recovery method from poultry environmental sample matrices.

Materials and methods

Sample collection for inoculation with *Salmonella*

Three separate trials were conducted using soiled foot covers and an additional trial was conducted using raw feed ingredients. On 3 separate occasions, 80 soiled foot covers were collected from one of three farms (80 from each farm). Farms were located in Arkansas, U.K. and Brazil. For the feed trial, 11 feed samples consisting of 8 germ meal and 3 soy meal were collected and pooled into a sterile plastic bag. The feed ingredients were mixed until a uniform distribution was accomplished. From this mix, three subsamples of 10 g were used in 3 separate PCR assays and confirmed as negative for *Salmonella*.

Salmonella inoculation and culturing conditions

For the foot cover trials, a frozen stock *Salmonella* Enteritidis strain was used that was previously recovered from the same farm sampled, with the exception of the U.K. farm because no *Salmonella* had previously been recovered from that farm. For the U.K. trial, a Ministry quality control *Salmonella* Enteritidis strain was utilized. Feed trials were performed with five replications, each replication with a different strain of *Salmonella*. Of the five strains utilized,

four strains, *S.* Kentucky, *S.* Berta, *S.* Tennessee, and *S.* Typhimurium var. Copenhagen, were previously isolated from feed mills. The fifth strain utilized, *S.* Enteriditis, was isolated from a broiler farm.

Foot cover trials

For foot covers, the 80 samples were randomly split into two different containers containing either sterile 1-X solution of the CVB buffered Peptone water (BPW) + Yeast Extract, or a 1-X solution of Tetrathionate (TT) with added Iodine solution and Brilliant Green solution 0.1% . For both BPW and TT, these samples were further split into aliquots of 100 mL. A frozen stock *Salmonella* culture was thawed and immediately serially diluted 10 fold to inoculate 100ul of the dilution into the aliquots of enrichment samples. All samples were incubated at 42^0C for 24 hrs. After this incubation period, samples were inoculated into MSRV and again incubated at 42^0C for 24 hrs. Finally, the MSRV samples were stuck onto Chromogenic / XLT4 agar using a sterile loop and all plates were incubated under the same conditions. For samples initially enriched in TT, the same procedure was used. After initial inoculation and after each 24 hour incubation, all samples were utilized in a real-time PCR assay. One bag of each set of the foot covers were not inoculated with *Salmonella* to act as negative controls.

Feed trials

For the feed samples, prior to splitting the sample and enriching in the two media, a 1000 g sub sample of the feed mixture was incubated in 5 L of sterile distilled water at 42^0C for 3 h with frequent agitation. The liquid then was filtered out through clean gauze foot swabs (Not

supplemented with dry milk). The liquid was split evenly into the BPW or TT Broth to make a complete 1-X solution of each enrichment media. Four 1 L volumes of the enrichment medias were then dispensed into forty wire closure 500 ml bags (100 mL volume per bag), and identified for the spiking trial with the various dilutions of the different *Salmonella* strains. Feed samples then were inoculated in the same manner as foot cover trials. Feed samples were incubated, passed and sampled in the same manner as described for the foot covers. One bag of each set of the feed was not inoculated with *Salmonella* to act as negative controls.

For samples enriched in BPW, a 1 mL portion of the sample was inoculated into 9 mL of TT and a 100 ul of the same sample was also enriched in MSRV. All samples then were re-incubated under the same conditions. Finally, the samples were streaked onto chromogenic/ XLT4 bi-plates using a sterile loop and all plates were incubated under the same conditions. For samples initially enriched in TT, the same procedure was used without the initial BPW pre-enrichment step. After initial inoculation and after each 24 hour incubation a portion of all the enriched samples were utilized in a real-time PCR assay.

DNA extraction and real-time PCR assays

At each enrichment step after incubation, a portion of the enriched sample was taken and utilized in a real-time PCR assay and a second sample was struck onto chromogenic / XLT4 agar bi-plates (Remel, Lenexa, KS). Any suspect colonies were collected from the agar and also evaluated by PCR. A 1 mL portion of the TT enriched sample was placed into a microcentrifuge tube and single colonies were placed in a microcentrifuge tube containing 1 mL of DEPC water

(Ambion, Foster City, CA). For MSRV samples, a 10 µl loop was suspended in 1 mL of DEPC water (Ambion). For all samples, the tubes were placed in a heating block and boiled at 101°C to lyse the cells and release the DNA. All samples were subsequently refrigerated at 4°C for 10 min. and finally centrifuged at 13,000 rpm for 2 minutes. All samples then were immediately used for the real-time PCR assay. The SYBR green real-time PCR assay was optimized using an Eppendorf Masterplex thermocycler ep (Eppendorf, Westbury, NY). Gradient Technology in the Eppendorf unit was used to optimize annealing and extension temperatures and times. The assay utilized published primers which targeted all serovars of *Salmonella* enterica (6) or *Salmonella enterica* serovar Enteriditis (2). The temperatures of the melting curves were 80.5ºC and 85ºC, respectively. All primers were synthesized by Sigma Chemical Company (St. Louis, MO). A 20 µl total volume reaction mixture consisted of 10 µl ml^{-1} of SYBR Green Premix Ex Taq™ (Takara; Fisher Scientific, Pittsburg, PA) 0.5 µmol ml^{-1} of each primer, 2 µl of DNA template and water to volume. The PCR reactions for both primers were optimized to the conditions of 94˚C for 1 min. then 35 cycles of 94˚C for 10 sec., 57˚C for 10 sec. and 72˚C for 20 sec. The threshold cycle number (Ct) was determined to be the cycle number at which fluorescence was greater than 400 of fluorescence units. Melting curves were created and analyzed with the Eppendorf realplex software (version 2.0).

Results

In this research, we conducted a series of 4 trials to determine the ability to detect *Salmonella* from poultry environmental matrices by real-time PCR and culturing. The first three trials were conducted using soiled foot covers collected from one of three farms (Arkansas, Brazil or U.K.). These materials collected from the covers were used as background matrices into which an SE culture was serially inoculated. In a second set of trials, feed components were utilized as the background matrix into which *Salmonella* were inoculated.

For foot cover trials using farm soils sampled in Brazil, the overall detection and recovery of *Salmonella* was more consistent between PCR and culturing when *Salmonella* was initially inoculated into TT versus BPW (Table 1 a,b). Furthermore, a 3 to 4 log difference in *Salmonella* detection by PCR was achieved when comparing samples incubated for one day in TT versus samples incubated for one day in BPW (Table 1 a,b). Similarly, the detection sensitivity with PCR was increased by 4 logs using TT with further enrichment in MSRV (Table 1B). Cultures were recovered after enrichment in TT and BPW with subsequent inoculation into MSRV (Table 1b), but the initial TT enrichment increased the sensitivity by one log. Finally, culture recovery on chromogenic / XLT4 bi-plates was increased by 4 logs when samples were inoculated into TT and enriched in MSRV versus initial inoculation into BPW.

For trials performed using soiled foot covers sampled from a farm in Arkansas, at least a 2 log difference in PCR detection was determined when comparing samples incubated overnight in TT versus BPW (Table 2 a,b). The same increase in PCR sensitivity was achieved after subsequent

enrichment in MSRV. A 1 log increase in recovery was acheived after further enrichment in MSRV when utilizing TT as the initial inoculum as opposed to BPW.

The final set of foot cover trials was performed using soiled foot covers sampled from a farm in the U.K. A 2 log increase in sensitivity with PCR detection was determined for initial enrichment in TT, but only for *Salmonella* specific primers (Table 3 a,b). There was no difference in PCR detection using the SE specific primers between samples inoculated into TT versus BPW. Interestingly, subsequent enrichment in MSRV after initial enrichment in BPW enhanced increased sensitivity by 1 log (Table 3b) over TT. A 1 log increase in MSRV sensitivity was achieved utilizing TT as the initial inoculum media versus BPW. There was a 1 log increase in final culture recovery on the plating media when samples were initially inoculated into TT versus BPW.

Interestingly, we noted a difference in the detection limit dependent on the PCR primer set utilized, the initial culturing medium and the background farm matrix. The samples from Brazil initially cultured in BPW resulted in a 1 log difference in detection limit difference with the two real-time PCR primers (Table 1a). The trials performed in the Arkansas and the U.K. had a 1 and 2 log difference, respectively, between the primer sets for those samples initially cultured in TT (Table 2b, 3b).

In the fourth trial, raw feed ingredients (soy meal and corn germ) were utilized as the background matrices and inoculated with one of five *Salmonella* enterica serovars (Kentucky, Typhimurium var. Copenhagen, Tennessee, Berta; Table 4a,b). After initial inoculation and 24

incubation in TT or BPW, the sensitivity of the PCR assay was increased by 1 to 4 logs depending on the serovar for inoculation in TT versus BPW. For some serovars, further enrichment in MSRV of samples initially inoculated into TT enhanced the sensitivity of PCR detection by 2 logs. None of the MSRV enrichments were positive for any serovar after initial inoculation in BPW. Cultures were recovered by plating MSRV enrichments onto chromogenic / XLT4 bi-plates, while inoculation into TT enhanced the detection sensitivity for some serovars by 1 log.

Discussion

Early detection of *Salmonella* contamination in poultry flocks is crucial to preventing colonization of the entire flock and possible cross-contamination among different flocks housed on the same facility. In this work, it was determined that PCR detection is possible after 24 hours of pre-enrichment within a solution of TT or BPW. However, in many cases it is necessary to obtain cultures as well as simple detection methods for further analysis such as serotyping or genotyping. This information can be useful in determining persistence of specific strains and to develop intervention or corrective measures.

This study demonstrated that detection limits by PCR and culturing were different when samples were initially pre-enriched in BPW as opposed to TT. This may be due to the enrichment of any bacteria present in the sample and *Salmonella* possibly being outcompeted for nutrients by other bacteria. As a result, the final titer of *Salmonella* may have been lower in the BPW than the TT samples. BPW is used as a pre-enrichment non-selective medium because it allows for repair of

cell damage and purportedly facilitates the recovery of *Salmonella* (5). Because BPW is buffered, the medium maintains a neutral pH which is important because stressed cells can be very sensitive to acidic conditions. TT is a selective enrichment medium and suppresses non-target organisms while allowing *Salmonella* to be cultured. Some inhibition of stressed cells has been reported when culturing in TT because TT is selective and *Salmonella* must overcome inhibitors aimed at suppressing non-target organisms (15). In these experiments, a frozen culture was thawed and immediately used to simulate stressed cells. However, the stresses encountered in a poultry environment would most likely be associated with desiccation or heat (11). Therefore, this may be a limitation of the study.

The differences in detection limits among samples taken from different locations may be due to several factors. First, background microflora would be expected to be different because climates among the farms were varied. As discussed, this background microflora can have an impact on the ability to recover cultures after enrichment. Secondly, variations in the soil chemistry among the farms could have impacted the ability to detect *Salmonella* by PCR. Chemicals present in soil, such as humic acid, are well known to inhibit PCR reactions (1,8). Finally, PCR inhibitors present in the samples may have affected the ability to detect *Salmonella*. In addition to soil chemicals, other inhibitors present such as those in fecal materials collected from the swabs may have affected the detection limits (14).

The result of differences in detection limits between primer sets was an interesting finding. In a few samples, the primers targeting SE had a lower detection limit than the *S. enterica* specific primers. Hoorfar et al. (6) reported inhibition of the primer set specific for *S. enterica* by *C.*

jejuni cultures. *C. jejuni* is extremely prevalent in the poultry environment (9). *C. jejuni* does not grow in TT, but can grow in BPW. Therefore, inhibition by *C. jejuni* may partially explain the differences in the sensitivity of the *S. enterica* primers but only in those samples cultured in BPW.

Although, many methods exist for isolating *Salmonella*, establishing a standard method is essential when multiple laboratories are comparing results. Standard methods allow data to be compared that is collected from independent laboratories and the development of databases against which future data can be compared. However, as demonstrated by our data, factors due to the environment that differ among laboratory locations can affect the results of an assay and this may be an inherent problem with standardization that needs to be taken into account. These experiments demonstrate that the sensitivity of *Salmonella* enhanced if samples are initially enriched in TT versus BPW. The detection of *Salmonella* is essential for control measures to be implemented. Considering the health burden of *Salmonella* foodborne illness, sensitive detection can be crucial to prevention of salmonellosis.

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Table 1. The detection of *Salmonella* enteric ser. Enteritidis (SE) from soiled foot swab samples initially enriched in buffered peptone water (BPW; panel A) or Tetrathionate (TT; panel B). A total of 80 foot covers were dragged through a farm in Brazil, randomly split into 2 different 2 L containers containing either BPW or TT and subsequently split into 100 mL samples that received a serially diluted culture of SE. After incubation, samples were further enriched in MSRV and finally struck onto chromogenic / XLT 4 agar. After each 24 h incubation, a portion of all enriched sample was utilized for a PCR assay using *Salmonella* specific primers (SAL) and *S.* Enteritidis specific primers (SE). Negative (-); Positive (+)

BPW

	Day 2 PCR		Day 3	PCR		Day 4
Inoculated $\log_{10}$ CFU mL^{-1}	SE	SAL	MSRV	SE	SAL	Chromogenic / XLT4 agar
0	-	-	-	-	-	-
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	+	+	-
6	-	+	-	+	+	-
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+

TT

Inoculated $\log_{10}$ CFU mL^{-1}	Day 2 PCR		Day 3 PCR			Day 4 Chromogenic / XLT4 agar
	SE	SAL	MSRV	SE	SAL	
0	-	-	-	-	-	-
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	+	+	-	+	+	+
4	+	+	-	+	+	+
5	+	+	-	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+

Table 2. The detection of *Salmonella* enteric ser. Enteritidis (SE) from soiled foot swab samples initially enriched in buffered peptone water (BPW; panel A) or Tetrathionate (TT; panel B). A total of 80 foot covers were dragged through a arm in the Arkansas, randomly split into 2 different 2 L containers containing either BPW or TT and subsequently split into 100 mL samples that received a serially diluted culture of SE. After incubation, samples were further enriched in MSRV and finally struck onto chromogenic / XLT 4 agar. After each 24 h incubation, a portion of enriched sample was utilized for a PCR assay using *Salmonella* specific primers (SAL) and *S.* Enteritidis specific primers (SE). Negative (-); Positive (+)

BPW

	Day 2 PCR		Day 3 PCR			Day 4
Inoculated $\log_{10}$ CFU mL^{-1}	SE	SAL	MSRV	SE	SAL	Chromogenic / XLT4 agar
0	-	-	-	-	-	-
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	+	+	+	+	+
7	-	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+

TT

	Day 2 PCR		Day 3 PCR			Day 4
Inoculated $\log_{10}$ CFU mL^{-1}	SE	SAL	MSRV	SE	SAL	Chromogenic / XLT4 agar
0	-	-	-	-	-	-
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	+	-	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+

Table 3. The detection of *Salmonella* enteric ser. Enteritidis (SE) from soiled foot swab samples initially enriched in buffered peptone water (BPW; panel A) or Tetrathionate (TT; panel B). A total of 80 foot covers were dragged through a farm in the U.K., randomly split into 2 different 2 L containers containing either BPW or TT and subsequently split into 100 mL samples that received a serially diluted culture of SE. After incubation, samples were further enriched in MSRV and finally struck onto chromogenic / XLT 4 agar. After each 24 h incubation, a portion of enriched sample was utilized for a PCR assay using *Salmonella* specific primers (SAL) and *S.* Enteritidis specific primers (SE). Negative (-); Positive (+)

BPW

	Day 2 PCR		Day 3	PCR		Day 4
Inoculated $\log_{10}$ CFU mL^{-1}	SE	SAL	MSRV	SE	SAL	Chromogenic / XLT4 agar
0	-	-	-	-	-	-
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	+	+	+
5	-	-	+	+	+	+
6	-	-	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+

TT

	Day 2 PCR		Day 3 PCR			Day 4
Inoculated $\log_{10}$ CFU mL^{-1}	SE	SAL	MSRV	SE	SAL	Chromogenic / XLT4 agar
0	-	-	-	-	-	-
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	+
4	-	-	-	+	+	+
5	-	+	-	+	+	+
6	-	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+

Table 4. The detection of 5 different serovars of *Salmonella enterica* from raw feed ingredients initially enriched in buffered peptone water (BPW; panel A) or Tetrathionate (TT; panel B). A total of 11 feed samples consisting of 8 germ meal and 3 corn germ meal were collected and pooled into a sterile plastic bag. A 1000 gram sub-sample was incubated in 5L of sterile distilled water at 42^0C for 3 h with frequent agitation, filtered out through clean gauze foot swabs (Non-milked) and randomly split into 2 different containers to make a 1X solution of BPW or TT and subsequently split into 100 mL samples that received a serially diluted culture of *Salmonella* . After incubation, samples were further enriched in MSRV and finally struck onto Chromogenic / XLT 4 agar. After each 24 h incubation, a 1 mL portion of enriched sample was utilized for a PCR assay using *Salmonella* specific primers.

BPW

	Day 2	Day 3		Day 4
Salmonella serovar	PCR limit of detection	MSRV limit of detection	PCR limit of detection	Plating limit of detection
S. Kentucky	2500	not detected	250	25
S. Berta	2.9	not detected	2.9	2.9
S. Typhimurium	30	not detected	30	30
S. Enteriditis	180	not detected	180	180
S. Tennessee	180	not detected	180	1.8

TT

	Day 2	Day 3		Day 4
Salmonella serovar	PCR limit of detection	MSRV limit of detection	PCR limit of detection	Plating limit of detection
S. Kentucky	0.25	2.5	2.5	2.5
S. Berta	0.29	2.9	2.9	2.9
S. Typhimurium	3	3	3	3
S. Enteriditis	1.8	1.8	1.8	1.8
S. Tennessee	0.18	1.8	1.8	1.8

Chapter Three

Detection of *Salmonella spp*. survival and virulence in poultry feed by targeting the *hilA* gene

Abstract

The objectives of this work were to evaluate immunomagnetic beads and a reverse transcriptase (RT) PCR method for detection of *Salmonella* inoculated into feed. In addition, a reverse transcriptase (RT) PCR method was evaluated for quantifying virulence gene *hilA* expression of *Salmonella* spp. in poultry feed matrices and utilized to determine the influence of poultry feed environmental factors on *Salmonella hilA* expression. An immunomagnetic separation technique was evaluated for increased recovery of *Salmonella* from feed. *Salmonella* cultures were inoculated into feed samples and exposed to heat treatments of 70 °C and sampled periodically. From these samples, RNA was collected and *hilA* gene expression was measured relative to the housekeeping 16S rRNA gene. The immunomagnetic bead protocol increased recovery by 1 log. The upregulation of *hilA* was demonstrated after 5 and 10 minutes of inoculated feed samples being exposed to heat treatment. From this work, the data indicate that the ability to detect live *Salmonella* cells in feed samples may be increased by targeting the *hilA* gene. Foodborne salmonellosis originating from poultry is a major problem and feed is a leading source of contamination in poultry, but detection in feed is complicated by low concentrations. The assays and experiments in this study examine possible improvements to recovery and detection of *Salmonella* in feed.

Introduction

Salmonella is the leading cause of foodborne illness in the United States with poultry and products implicated in 29% or approximately 406,000 cases annually (2,16). The costs associated with non-typhoidal *Salmonella* infections are estimated at nearly $2.4 billion dollars annually, due to loss of productivity and medical treatment costs (27). In addition, poultry producers may incur losses due to *Salmonella* infection of the flock such as, decreased performance, loss of birds and production time. These losses in the United States per year have been calculated to be approximately $64 million - $114 million, however calculations do not take into account the loss of eggs and other consumable poultry products.

Young chicks are more susceptible to infection with *Salmonella* than mature birds due, in part, to a lack of protective gut microflora (12,24). For this reason, 1-day old chicks can be colonized with as few as 5 cells of *Salmonella*, but colonization of 2 week old birds which have protective microflora is inconsistent and requires higher doses (21). The use of antibiotics to control *Salmonella* in poultry is not an option and alternatives to antibiotics for control of bacteria in poultry such as bacteriophage and probiotics have yet to be completely successful (11,22). For these reasons, control through sampling and monitoring programs is essential to reduce potential infection.

Infection of a flock with *Salmonella* can spread rapidly with the entire flock becoming infected within 2 to 10 days (3). Furthermore, *Salmonella* is capable of remaining in a poultry environment between flocks (5,26). *Salmonella* may be introduced to a flock by multiple

sources, but poultry feed has been demonstrated to be a leading source (17). Detecting *Salmonella* in feed can be challenging because low levels of the bacteria that may not be recovered using traditional culturing and sampling techniques. Numerous detection methodologies, cultural and molecular based, have been examined over the years for quantifying *Salmonella* in feeds and some have proven to be more effective for *Salmonella* recovery (10,18,19).

Given the need for increased detection sensitivity, molecular detection technologies may be the best approach for identifying small numbers of *Salmonella* in large volumes of feed. The primary difficulty with molecular assays is the problem of extracting and recovering representative samples from feeds for molecular analyses. Although standard PCR is sufficiently sensitive to detect specific DNA sequences present in a sample, DNA does not distinguish whether the *Salmonella* are alive and functioning or dead. In addition, DNA reveals little about the virulence and viability of *Salmonella* and its potential to infect animals such as poultry and humans. Therefore, the objective of this study was to develop and test a molecular method that could detect and characterize viable *Salmonella* in feed.

Materials and methods

Bacteria and culturing condition

For these experiments, *Salmonella* Typhimurium (wild type isolated from a poultry farm) and *Salmonella* Seftenberg (wild type isolated from feed) were used. *Salmonella* was cultured on

Tryptic Soy Agar (TSA, EMD Chemicals Inc, Gibbstown, NJ, USA) and plates were incubated at 37˚C for 24 h. Prior to use in all experiments, the cultures were suspended in PBS and enumerated by using a 100 µL portion of the suspension in a dilution series.

DNA and RNA extraction

Genomic DNA was extracted from a 1 mL suspension of cells in PBS by boiling in a water bath for 10 min and subsequent refrigeration at 4°C for 10 min. The samples were then centrifuged at 16,000 x g for 2 min and a 100 µL of the supernatant was used for template DNA. All samples were immediately used in PCR assays. For RNA preparation, a 1:1 volume of Trizol reagent (Sigma, St. Louis, MO, USA) was added to the sample and extraction of RNA then was performed with the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (Valencia, CA, USA).

Conventional PCR assay

The PCR assay was optimized using a MJ PTC 100 thermocycler (Bio-Rad, Hercules, CA, USA). Primer sets for the PCR assay were previously published by Weir *et al.* (25). All primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). The reactions resulted in a 146-bp fragment for the 16S rRNA primer set and 130-bp fragment for the *hilA* primer set. Reactions specific for each primer set were done independently and each reaction consisted of a 25 µL total volume mixture with 12.5 µL of SYBR Green Premix Ex Taq^{TM} (Takara, Fisher Scientific, Pittsburg, PA, USA), 400 nM of each primer, 1 µL of DNA template

and water to volume. The PCR reaction was optimized to the conditions of 94 ˚C for 2 min. then 35 cycles of 94 ˚C for 30 sec., 55 ˚C for 30 sec. and 72 ˚C for 30 sec. with a final extension cycle at 72˚C for 5 min. The PCR products were electrophoresed in a 2 % agarose gel at 100 V for 20 min. Gels were stained with ethidium bromide (10 mg/mL) and viewed with a UV transilluminator.

Reverse-Transcriptase Real-Time PCR assay (qRT-PCR)

The qRT-PCR assay was optimized using an Eppendorf Masterplex thermocycler ep (Eppendorf, Westbury, NY, USA). Gradient Technology in the Eppendorf unit was used to optimize annealing and extension temperatures and times. The same primer sets utilized for conventional PCR also were used for the qRT-PCR reaction. A 20 µL total volume reaction mixture consisted of 10 µL of EXPRESS SYBR Green ER^{TM} qPCR SuperMix with Premixed ROX (Invitrogen, Carlsbad, CA, USA), 0.5 µL of EXPRESS SuperScript Mix for One-Step SYBR Green ER (Invitrogen), 500 nM of each primer, 100 ng of RNA template and water to volume. The qRT-PCR reaction was optimized to the conditions of 50 ˚C for 5 min for the initial reverse transcriptase step. This was followed by 40 cycles of 95 ˚C for 15 sec., 55 ˚C for 15 sec. and 68 ˚C for 20 sec. with fluorescence being measured during the extension phase. Melting curves were done subsequently and consisted of 95 ˚C for 15 sec., 60 ˚C for 20 min increasing by 0.5˚C per min to a final temperature of 95 ˚C. For gene expression analysis, samples were normalized using the 16S rRNA gene as an internal standard. Cycle threshold values were determined with the Eppendorf realplex software (version 2.0). The relative changes (*n*-fold) in *hilA* transcription

between the treated and non-treated samples were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001).

Construction of the qRT-PCR standard curve

To create the standard curve assay and determine the limit of detection for the qRT-PCR, serial dilutions were prepared from RNA obtained from a culture with a known initial cell concentration determined from a dilution series. The fluorescence along with the RNA template concentration results were used to construct a linear curve that correlated the first cycle number at which fluorescence was detected to the number of cells per mL. For each reaction, the threshold cycle number (Ct) was determined to be the cycle number at which fluorescence was greater than 400 fluorescence units. The efficiency of the reactions were calculated with the formula $E=10^{(-1/slope)} -1$. Melting curves were created and analyzed with the Eppendorf realplex software (version 2.0).

Spiking and analysis of feed samples

Feed samples collected from a local co-op store were utilized for this portion of the study. All samples were analyzed for the presence of *Salmonella* using conventional plating techniques. Initially, a 25g portion of the feed sample was suspended in 50 mL of tetrathionate broth supplemented with iodine solution. The suspension was incubated at 37 ˚C for 24h. The suspension was then streaked onto XLT4 agar and incubated under the same conditions as the tetrathionate suspensions. To spike feed samples for analysis, a 200 mL suspension of

Salmonella in PBS was initially prepared and from this suspension, 30 mL aliquots were inoculated into 15 g of feed. Feed matrices were exposed to 70 ˚C and sampled at periodic times of 0, 5, 10, 15 and 20 min. and 1 mL portion of the samples were used for RNA extraction.

Immunomagnetic separation of Salmonella

Anti- *Salmonella* Dynabeads (Invitrogen) were used to separate *Salmonella* spp. from feed matrices and this approach is based on an immunomagnetic separation technique previously used to remove *Salmonella* Enteritidis from feces and cecal samples (6). A dilution series was prepared from a 50 mL suspension of *Salmonella*. This dilution series was used to inoculate 8 separate 25 g feed samples. After incubation at room temperature for 2 h, 1 mL triplicate samples were plated on XLT4 agar to enumerate *Salmonella*. In addition, triplicate 1 mL portions of feed samples were placed into 1.5 mL sterile microcentrifuge tubes with 20 µL of anti- *Salmonella* Dynabeads. The microcentrifuge tubes were transferred to a Dynal MPC-M rack and samples processed according to the manufacturer's instructions. Dynabeads-bacteria complexes were resuspended in 1 mL of bacteria protect reagents (Qiagen, Valencia, CA, USA) and RNA extracted from the samples.

Feed mill trial

Approximately 50 lbs. of cracked corn was obtained from the local co-op. Corn was sieved through a screen (No. 8; 2.38 mm openings) to remove dust and small particles. The *Salmonella* Seftenburg suspension was prepared in a 1 L flask of PBS. Green food coloring was added to the

PBS to track the corn through processing. The 1 L flask of suspension was added to the corn and mixed thoroughly. On the day of the trial, a 25 g portion of the corn was taken to determine the CFU per g of corn by suspending a 1:9 ratio of corn into PBS in a sterile plastic bag. The suspension was massaged by hand for 2 min and a dilution series performed for enumeration. At the feed mill, inoculated corn was added to the feed mill process at a "hand-add" station. The "hand-add" station is a port in the system prior to the steam processing, but before the pelleting station where additional components or dyes can be added to the mixture of raw ingredients. All 4 tons of processed feed were collected into a feed tanker and the feed distributed into totes. The feed was collected and pellets of feed containing dyed pieces of corn were analyzed. Feed samples were analyzed by culturing as well as using DNA and RNA in the PCR based assays.

Results

Conventional PCR

An ultimate goal of this project was to utilize RNA as a target for detection of viable *Salmonella* in feed. The first step in accomplishing that goal was to utilize DNA to optimize PCR conditions more efficiently and with a lower cost. The primer pairs for both the 16S rRNA and *hilA* genes were successful at amplifying the target genes. These primer pairs produced a PCR product that was 146-bp and 130-bp in size for the 16S rRNA and *hilA* genes, respectively.

Limit of detection and standard curve

A main problem with detecting *Salmonella* in feed is the low levels of cells present in large quantities of feed. Thus, the detection limits of the RT-PCR assay and an experiment to determine the efficacy of imunomagnetic beads to concentrate and collect *Salmonella* cells from a feed matrix were performed. To optimize and determine the number of cells necessary to detect RNA using the RT-PCR assay, the reverse transcriptase PCR assay was performed using a dilution series of RNA. The limit of detection using this assay was 490 cells for both the *hilA* and 16S rRNA primer sets (Figure 1). The melting curves for *hilA* and 16S rRNA were 84 and 87^0C, respectively.

For immunomagnetic bead experiments, feed samples were inoculated with a dilution series of a prepared *Salmonella* suspension in PBS and collected with immunomagnetic beads. The limit of detection with the Dyna beads was 500 cells inoculated into 25g of feed. With the inoculum to feed ratio, this would mean each gram would have approximately 25 *Salmonella* cells. Since a 1 g portion was used, the true limit of detection then was actually 25 cells per gram. Compared to the limit of detection from direct plating which was 100 cells per gram.

Analysis of gene expression

Experiments were performed to simulate heat treatment of feed artificially contaminated with *Salmonella* to determine any up-regulation in the virulence gene regulator *hilA*. In these experiments, *hilA* was up-regulated at least 1.3-fold after 5 min. at 70°C (Table 1). Increase in *hilA* expression continued at 10 minutes. The expression levels of *hilA* were also measured after 15 and 20 minutes. However, RNA recovered after this amount of time was unreliable due to low quantity and quality and thus these data points were not considered reliable.

Feed Mill Trial

A feed mill trial was also done in order to assess the survival ability of *Salmonella*. For these experiments a wild type *Salmonella* Seftenburg was used for two reasons: 1) it was originally isolated from feed; and 2) *Salmonella* Seftenburg has been demonstrated to have increased survival ability over other serovars due to stresses including heat and desiccation (Liu *et al.* 1969; Kumar and Kumar 2003). Samples were taken immediately after processing of the feed for analysis. From the samples, *Salmonella* DNA was recovered and PCR utilizing DNA from feed samples were PCR positive. All samples subjected to culturing and reverse-transcriptase PCR utilizing RNA were negative.

Discussion

Detection of *Salmonella* in feed can be challenging due to a low number of cells present in a large volume of feed. Riley (23) estimated a contamination rate of feed passing through a contaminated cooler would pick up 1 *Salmonella* organism per 10 to 100 tons if the facility was not receiving feed ingredient loads that were contaminated. At such a level of contamination, the challenge becomes designing both a sampling program and a method of detection that can detect 1 cell in 10 tons of feed.

We utilized immunomagnetic beads to capture bacteria and tested whether this method improved recovery of *Salmonella* from feed samples. With this method, an antibody linked to a magnetic

bead captures the *Salmonella*. The beads are then collected with a magnet and the remaining sample can be washed away. Captured particles are finally released from the immunomagnetic complex. The limit of detection with the Dyna beads was 490 cells inoculated into 25g of feed. This would mean that theoretically each gram would have to have 20 *Salmonella* cells for detection. Since a 1 g portion was used, the true limit of detection then was actually 20 cells per gram. In addition, the qRT-PCR assay used in this study had a limit of detection of 490 cells to provide enough RNA for detection. Although the immunomagnetic bead method improved the recovery of *Salmonella*, it is still not sensitive enough to detect the estimated 1 cell in 10 tons of feed. Thus, further research and improved sampling procedures will be needed.

A second aim of this research was to develop an RNA-based PCR assay that could detect and quantify gene expression of *Salmonella hilA*. The virulence regulator *hilA* is a key gene for colonization and thus up-regulation of this gene can lead to enhanced colonization or organ invasion (1,7). The *hilA* gene has been demonstrated to be up-regulated due to stresses encountered such as acid stress (8). In addition, Churi *et al*. (2010) demonstrated an increase in *hilA* expression after exposure to mild heat stress (45^0C). Therefore, we hypothesized that heat stress encountered during processing of feed would increase the virulence gene *hilA*. Our data did in fact show that *hilA* was up-regulated after exposure to 70 °C after 5 min and increased consistently after 10 minutes.

The RNA based assays are advantageous because they are more rapid than culturing and distinguish between live and dead cells. In recent years, the number of real-time PCR assays for the detection of *Salmonella* in food and other matrices has increased (9,20). However, a

disadvantage is the fact that RNA rapidly degrades. These disadvantages might be overcome by initially increasing the amount of available RNA and this might be accomplished when target genes are upregulated.

Our data indicates that the *hilA* gene of *Salmonella* is up-regulated when inoculated into feed and exposed to heat stress. Thus samples of feed taken immediately after processing and treated with RNA preservation reagents could provide enhanced recovery of viable *Salmonella* cells when utilizing the RNA assay developed in this work. Unfortunately, our feed mill trials did not recover any viable *Salmonella* and therefore this will have to be validated at a later time. We hypothesized that because the *Salmonella* cultures we utilized in the feed mill trials was not previously subjected to heat stress, that the cells may have been more susceptible to heat stress. Therefore, modifications in the inoculating procedure need to be made in order to more closely simulate the physiology of the *Salmonella* that might be found in non-artificially inoculated feed samples. A caveat of these experiments is that only one strain of two *Salmonella* serotypes was utilized. Thus, caution should be taken when applying the results of this work to other strains and serovars of *Salmonella*.

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Table 1. Results of the *hilA* gene expression after *Salmonella* was inoculated into feed samples and exposed to heat treatment. Triplicate samples were taken and RNA collected at times points 0, 5, and 10 mins. post heat treatment performed in three independent trials.

TRIAL 1

Sample Time	*hilA* Ct	16S rRNA Ct	ΔCt	Mean	St. dev.	$^{\Delta\Delta}$Ct	$2^{-\Delta\Delta Ct}$	Fold Change
0 min	24.93	10.01	14.92	15.05	0.113	0	1	
	25.34	10.23	15.11					
	25.53	10.41	15.12					
5 min	25.25	10.28	14.97	14.64	0.384	-0.41	1.32	1.32 fold increase
	25.34	10.6	14.74					
	25.49	11.27	14.22					
10 min	23.88	10.58	13.3	13.16	0.361	-1.89	3.71	3.71 fold increase
	23.93	10.5	13.43					
	24.08	11.33	12.75					

TRIAL 2

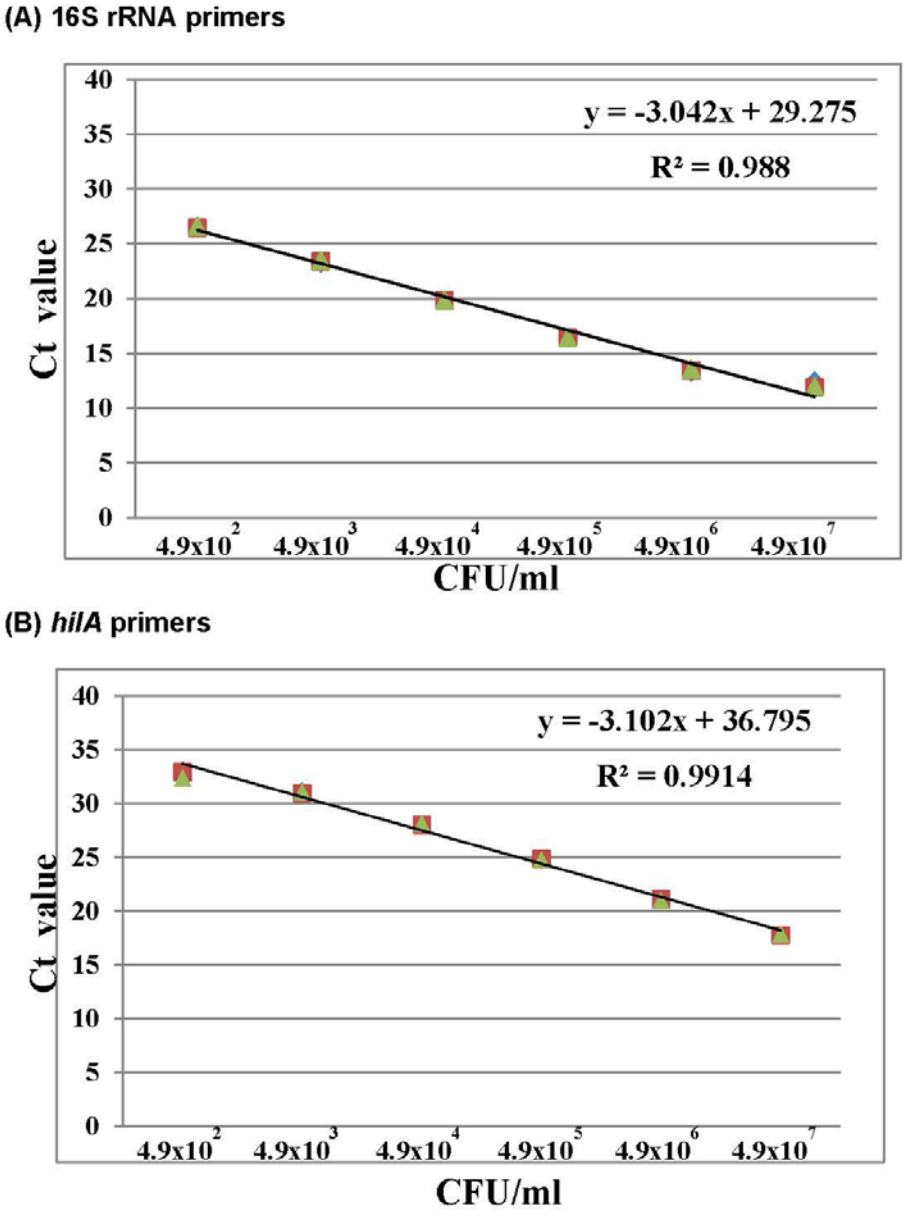
Sample Time	*hilA* Ct	16S rRNA Ct	ΔCt	Mean	St. dev.	$^{\Delta\Delta}$Ct	$2^{-\Delta\Delta Ct}$	Fold Change
0 min	26.37	10.6	15.77	15.31	0.434	0	1	
	26.57	11.66	14.91					
	26.75	11.51	15.24					
5 min	25.63	10.67	14.96	14.62	0.504	-0.69	1.61	1.61 fold increase
	25.89	11.03	14.86					
	25.69	11.65	14.04					
10 min	23.78	10.82	12.96	13.12	0.408	-2.19	4.56	4.56 fold increase
	23.86	11.05	12.81					
	24.06	10.48	13.58					

TRIAL 3

Sample Time	*hilA* Ct	16S rRNA Ct	ΔCt	Mean	St. dev.	$^{\Delta\Delta}$Ct	$2^{-\Delta\Delta Ct}$	Fold Change
0 min	25.83	10.38	15.45	15.36	0.272	0	1	
	26.25	10.68	15.57					
	26.3	11.25	15.05					
5 min	24.12	11.01	13.11	12.99	0.437	-2.36	5.15	5.15 fold increase
	24.1	10.74	13.36					
	24.14	11.63	12.51					
10 min	22.66	10.2	12.46	12.16	0.389	-3.19	9.17	9.17 fold increase
	22.59	10.29	12.3					
	22.57	10.85	11.72					

Figure 1.

A standard curve of the 16SrRNA PCR primers (A) and the *hilA* PCR primers (B) from the reverse-transcriptase real-time PCR assay (qRT-PCR) utilizing RNA.



Chapter Four

Development of a real-time polymerase chain reaction for the simulataneous detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*

Abstract

In this research we developed a real-time SYBR green assay to detect both *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in a single reaction. A total of 30,000 samples from broiler breeder flocks were screened using traditional serology (plate agglutination, ELISA, hemagglutination inhibition) and PCR (traditional and real-time). It was determined that the real-time SYBR green PCR assay developed in this research was more rapid than all three methods tested and more sensitive and specific than culturing or serology. The SYBR green assay was optimized and could detect as few as 30 template copies of DNA per sample. In addition, the SYBR green assay was less expensive than traditional culturing and serology. *Mycoplasma gallisepticum* and *M. synoviae* are infectious bacteria that can rapidly spread and infect commercial chicken flocks. These diseases can cause a significant loss to the poultry industry and especially broiler breeders because infected flocks are destroyed under the National Poultry Improvement Plan (NPIP) MG and MS clean programs. The real-time SYBR green assay developed in this research has the potential to reduce the time of reaching a correct diagnosis and arrest outbreaks of MG and MS.

Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are the infectious agents of chronic respiratory disease in chickens. Clinical symptoms include coughing, nasal discharge, and air sac lesions, but in some infections no clinical symptoms appear (4). Chickens may be inoculated with as few as 100 cells and start excreting the bacteria as soon as 3 days post inoculation (4). Both horizontal and vertical transmission has been shown to occur rapidly (17).

Mycoplasma gallisepticum and *M. synoviae* are two common poultry pathogens that continue to significantly impact breeder, broiler, and egg laying production. *Mycoplasma* infects approximately 90% of U.S. egg laying birds and reductions in egg production are estimated at $140 million annually (14). In broilers, a reduced feed conversion efficiency, depressed growth rate, and condemnation of carcasses can be economically devastating. Losses as high as $750,000 have been reported from a single outbreak of MG (3).

Mycoplasmas are treatable with antibiotics. However, treatment may not be fully effective at clearing the infection (6, 16). Eradication of infected flocks in most instances is the solution. Stringent biosecurity and biosurveillance practices which can facilitate early intervention strategies are necessary to control *Mycoplasma* infections. Diagnostic methods for detecting *Mycoplasma* infection typically used may include culture, serology or molecular assays. Traditional culturing is the least favored because the method is time consuming, the bacteria are slow growing, and some fastidious strains may not be detected (2). Serology is much faster than culturing, but non-specific reactions and cross-reactions between species, mis-interpretations due

to recent vaccination, and cost are all disadvantages (5). In addition, antibodies to MG and MS may not be detected until 1 to 3 weeks post-infection (9).

Polymerase chain reaction (PCR) assays can be used as detection methods with results in as few as 24 h. Real-time PCR has additional advantages over traditional PCR including: 1) real time is more rapid and can be accomplished in as little as 40 minutes; 2) No post amplification processes are required which decreases total detection time, cost in terms of materials, and hazardous waste; 3) are more sensitive - some real time assays can detect as few as 10 template copies per 5µl sample; 4) questionable results can be confirmed using melting curves. The objective of this study was to develop a SYBR green real-time PCR assay and compare the real-time PCR assay with conventional PCR, traditional culturing and serology to determine the most rapid, cost-effective, sensitive and specific method for the detection of MG and MS under industry conditions.

Materials and Methods

Sampling for Mycoplasma

Approximately 200 broiler breeder flocks located in Arkansas, Oklahoma, Missouri and Georgia were sampled once every three weeks. Sample collections consisted of 15 blood samples per air space for serology and 15 palatine cleft swabs per air space for PCR detection. During the trial period (November 2006 to May 2007) a total of 30,000 palatine cleft swabs and 30,000 blood samples were collected and analyzed.

Bacteria and culturing conditions

If a positive sample was detected in a flock by serology (plate agglutination and ELISA) and PCR (conventional and SYBR green real-time PCR), a second sampling of palatine cleft swabs was done as described in the previous section. In addition, tracheal samples were collected by euthanizing a representative sample of the affected flock (approximately 30 tracheal samples per 6000 birds) and evaluated by culture. An upper portion of the tracheas were aseptically removed and collected in sterile containers. Tracheas were subsequently frozen at -74°C and shipped the following day on dry ice (a portion of each tracheal sample was retained as a backup sample). All tracheas were sent to the Poultry Disease Research Center (PDRC), Athens, GA for analysis by culturing using published methods (10). If cultures were grown from the tracheal samples, PCR confirmation and sequencing of the *mgc2*/IGSR and *vlhA* genes for MG and MS, respectively, were preformed according to previously published methods (8,15)

Serology assay

Serology assays were conducted by measuring antibody levels in chicken serum to MG and MS using both plate agglutination and ELISA assays. Chicken blood samples were collected in low adhesion microcentrifuge tubes (1415-2600; USA Scientific, Ocala, FL), from which the serum could be easily separated from the clot naturally after holding at room temperature (25°C), overnight. Plate agglutination assay was performed as previously described (10). Positive and negative control serums (Charles River) also were used to compare against the sample. Any

positive plate agglutination samples were further tested using the IDEXX enzyme immunoassay (IDEXX, Westbrook, MA) according to the manufacturer's instructions. In addition, duplicate blood samples were sent to the appropriate state approved NPIP laboratory at which plate agglutination and hemagglutination inhibition (HI) serology assays were performed for detection of MG and MS.

DNA extraction for PCR and real-time PCR assays.

Genomic DNA was extracted from the samples and positive controls using a previously published method (11) with some modification. Briefly, the palatine cleft swab samples were pooled into groups of 5 swabs. DNA was extracted by placing swabs from palatine cleft samples in 1 ml of phosphate buffered saline (PBS;Gibco®, Invitrogen, Carlsbad, CA) and twisting the swab vigorously. Excess fluid from the swab was released by pressing the swab against the wall of the tube with PBS. The sample was then centrifuged at 13,000 rpm for 2 min. The supernatant was discarded and the pellet was finally suspended in 50 ul of Diethyl Pyrocarbonate treated water (DEPC; Sigma, St.Louis, MO) to reduce nuclease activity. The tubes were placed in a heating block and boiled at 101°C to release the DNA from the cells. The samples were subsequently refrigerated at 4°C for 10 min. and finally centrifuged at 13,000 rpm for 2 minutes. All samples were then immediately used for PCR assays. DNA quantifications showed an average DNA template between 80 to 100 ng μl^{-1}. For positive controls, DNA was prepared from hemagglutination antigen (HA; USDA National Veterinary Services Laboratory, Ames, IA) specific for MG and MS. A 100 µl sample of HA was suspended in 900 µl of PBS containing 50 µl of DEPC. The samples were centrifuged and boiled as described for swab samples prior to

use. For negative controls, DNA from palatine cleft swabs of a pool of 300 *Mycoplasma* negative chickens was prepared as described in this section.

PCR assay

The PCR assay was optimized using a MJ PTC 100 thermocycler (Bio-Rad, Hercules, CA). The PCR assay utilized primers previously published (11, 12) with some modifications. Primer sets utilized for the *M. synoviae* reaction were MSLF (5'GAG AAG CAA AAT AGT GAT ATC A 3') and MSLR (5'CAG TCG TCT CCG AAG TTA ACA A 3') and for the *M. gallisepticum* reaction were MG-14F (5'GAG CTA ATC TGT AAA GTT GGT C 5') and MG-13R (5' GCT TCC TTG CGG TTA GCA AC 3'). The reactions resulted in a 186 bp fragment for MG and 214 bp fragment for MS. Reactions specific for MG and MS were done independently and each reaction consisted of a 25 µl total volume mixture with 12.5 µl ml^{-1} of RedTaq Ready Mix (Sigma Aldrich, St. Louis, MO), 0.5 µmol ml^{-1} of each primer, 3 µl of DNA template and water to volume.

SYBR green real-time PCR assay

The SYBR green real-time PCR assay was optimized using an Eppendorf Masterplex thermocycler ep (Eppendorf, Westbury, NY). Gradient Technology in the Eppendorf unit was used to optimize annealing and extension temperatures and times. The same primer sets utilized independently for conventional PCR, listed above, were combined and used in a single SYBR green real-time PCR reaction. All primers were synthesized by Sigma Chemical Company (St.

Louis, MO). A 20 µl total volume reaction mixture consisted of 10 µl ml^{-1} of SYBR Green Premix Ex Taq™ (Takara; Fisher Scientific, Pittsburg, PA) 0.5 µmol ml^{-1} of each primer, 2 µl of DNA template and water to volume. The PCR reaction was optimized to the conditions of 94˚C for 1 min. then 35 cycles of 94˚C for 10 sec., 57˚C for 10 sec. and 72˚C for 20 sec. If a positive sample was detected, a melting curve and confirmatory reactions using the primers MG and MS independently were done to determine the presence of either MG or MS. The reaction mixture was modified to a 20 µl total volume reaction mixture consisting of 10 µl ml^{-1} of, SYBR Green Premix Ex Taq™ (Takara, Fisher Scientific) 0.5 µmol ml^{-1} of each primer, 2 µl of DNA template and water to volume. The reaction conditions remained the same as described earlier.

Construction of SYBR green real-time PCR standard curve

To create the standard curve for the SYBR green PCR assay, serial dilutions of DNA were prepared from HA specific for MG and MS as described in the previous section. Each DNA sample from the dilution series was quantified using a fluorimeter and template copy numbers were calculated based on the genome size of MG and MS using a previously published method (7, 13, 18). The fluorescence along with the DNA template number results were used to construct a linear curve that correlated the first cycle number at which fluorescence was detected to the number of template copies of DNA. For each reaction, the threshold cycle number (Ct) was determined to be the cycle number at which fluorescence was greater than 400 of fluorescence units. The efficiency of the reactions were calculated with the formula $E=10^{(-1/slope)}$ -1. Melting curves were created and analyzed with the Eppendorf realplex software (version 2.0).

Results

In this study, a total of 30,000 blood samples and 30,000 palatine cleft swab samples were analyzed for MG and MS (Table 1). Serum from each blood sample was analyzed on an individual basis and of these 30,000 samples, 1980 (6.6%) were positive using plate agglutination. Of the 1980 positive samples, only 44 were positive using the ELISA samples. All samples tested with plate agglutination and HI at the appropriate state approved NPIP laboratory correlated with the results of the ELISA testing done at our laboratory. The palatine cleft swab samples were pooled into groups of 5 swabs and analyzed using the real-time SYBR green PCR assay developed in this study. Of the 30,000 samples tested 280 were positive for MG or MS.

In the time frame of this study, a total 13 cases of *Mycoplasma* occurred. A single case was defined as an incident at a single production site beginning with a positive sample, followed by positive re-sample, corrective actions taken and ending with all negative re-samples in the production site. Of the 13 cases, 12 were MS and 1 was MG as determined by the SYBR green real-time PCR assay and confirmed with conventional PCR. Only 7 of the 13 cases were detected using serology. Tracheas sent for culturing obtained bacterial cultures for 8 of the 12 MS positive samples and for the 1 MG positive sample. The 4 cases that were negative for culturing occurred within a one month or less time frame on the same production site as previous cases that were positive for culturing. However, we chose to call these 4 cases separate for two reasons 1) there was a one month or less lag phase between the cases where all PCR and serology samples tested negative and 2) sequencing of genes to positively connect the cases with the same infecting strain was not possible due to negative cultures.

The detection limits of the SYBR green real-time PCR assay was determined by using the threshold (Ct) values from three independent reactions (Figure 1). For MG, the assay was able to detect 27 template copies of DNA per sample (Figure 1A). For MS, the assay was able to detect 28 template copies of DNA per sample (Figure 1B). A threshold value above background fluorescence was chosen and verified using duplicate negative controls (Figure 2). The efficiencies of the reactions (the ability of the reaction to double the PCR product with each cycle) were calculated as 93.4% for MG and 106.6% for MS. The melting temperatures of the amplicons (Tm) for MG and MS were determined to be 85.7 and 85.0°C, respectively (Figure 3).

Discussion

In this assay we were able to combine two sets of primers (MG and MS) in a SYBR green real-time PCR assay. Since SYBR green binds to all double stranded DNA, primer dimmers and non-specific products may also produce a fluorescent signal. Therefore, the PCR primers must be very specific. The two primer sets utilized in this work were previously published with a known sensitivity of 70 to 100 cfu ml^{-1} (11, 12). In addition, these primer sets are widely used in the poultry industry and are approved for detection of MG and MS by the NPIP. If any non-specific binding were in question, an analysis of the melting curve produced by the computer program during the reaction could be viewed to determine any error.

Both MG and MS are considered serious problems and warrant further investigation regardless of the infecting species. Therefore, we were able to disregard the distinction between MS and

MG in our assay and combine the two primer sets into one reaction. This would have been more difficult with conventional PCR because the two amplicons are 28 bps in difference. Distinguishing these sizes by electrophoresis would require adjusting the electrophoresis conditions which would add more time to the PCR assay. However, the melting curve analysis from the real-time SYBR green assay relied on the GC content of the amplicon and its base per content and is therefore more accurate than distinguishing PCR products by size. Sensitivity of the assay developed in this work was also a concern, but using a Taq-man probe specific for the detection of only MG Callison and co-workers (1) had almost the same sensitivity as our reaction.

A total of 3 hours (including extraction time) were needed to perform the real-time PCR assay developed and utilized in this study. However, the plate agglutination serology test took 24 hours because the blood samples were allowed to separate before serum could be used. The ELISA assay took a total of 31 hours to complete. Culturing was the longest and most labor intensive of the assays utilized in this study and required a total of approximately 3 to 4 weeks for results.

Detectable antibody titers may not be present until 1 to 3 weeks post infection which made serology the least sensitive of the assays compared in this study. Culturing was also less sensitive than the PCR based assays. Of the 13 cases that tested positive by PCR, culturing was positive for only 9 cases. This may be because traditional culturing failed to detect any injured or stressed cells, freezing of the trachea samples may have killed low numbers of *Mycoplasma* and / or other organisms quickly outcompeted *Mycoplasma*. DNA based assays are more sensitive. However, some DNA may be lost during the preparation when certain kits are utilized, because multiple

steps are typically required and each step may result in the loss of some DNA. Lysing the cells by boiling and rapidly cooling to release the DNA was found to be very rapid and conserved the amount of DNA template in this study. Furthermore, the expense and time of using a kit was avoided.

In addition to speed, cost can be a large factor in utilizing any assay. Since PCR has become a common technique used in most laboratories, the equipment utilized including thermocyclers is usually available. The remaining cost including primers, reagents and consumables (tubes, tips, and other disposables) of the PCR assay described in this study is approximately 0.65 dollars per reaction. This would make the cost of the PCR reaction about the same as the cost of traditional culturing and far less expensive than the commercially available serology assay used in this study. Since a thermocycler equipped to detect fluorescence signal was used for the real-time PCR reactions, this initial investment can be somewhat costly. However, the increasing popularity and acceptance of fluorescence based PCR is leading to this equipment becoming more affordable. In addition, the cost of the reagents used in the fluorescence based assay described in this study cost the same as than the reagents used in the traditional PCR assay. Overall, the SYBR green assay developed in this work was the most rapid and inexpensive of all the methods used.

With this particular assay, exact quantification of an unknown sample was not possible. The primers used in this study target the 16sRNA gene and there are two copies of this gene in each cell. For this reason, one or both copies of the gene might be amplified in the initial cycles.

Therefore, the standard curves created in this study can be used to calculate reaction efficiency but cannot be used to quantify an exact number of CFUs for unknown samples.

Infection of broiler breeder flocks with MG or MS can be economically devastating to producers. The real-time PCR surveillance tool developed in this research has the potential to save producers from these large losses because of a reduced detection time allowing producers to act quickly and prevent spread of disease. Furthermore, the assay developed in this research was shown to be highly sensitive and specific for the detection of both MG and MS.

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Figure 1. Calibration curve of real-time PCR for *Mycoplasma gallisepticum* (Panel A) and *Mycoplasma synoviae* (Panel B) reactions. Curves were created by plotting 10-fold serial dilutions of prepared *Mycoplasma* DNA versus the cycle threshold (Ct) value. The values of the X-axis are expressed as 10^n , where n = the fold of dilution.

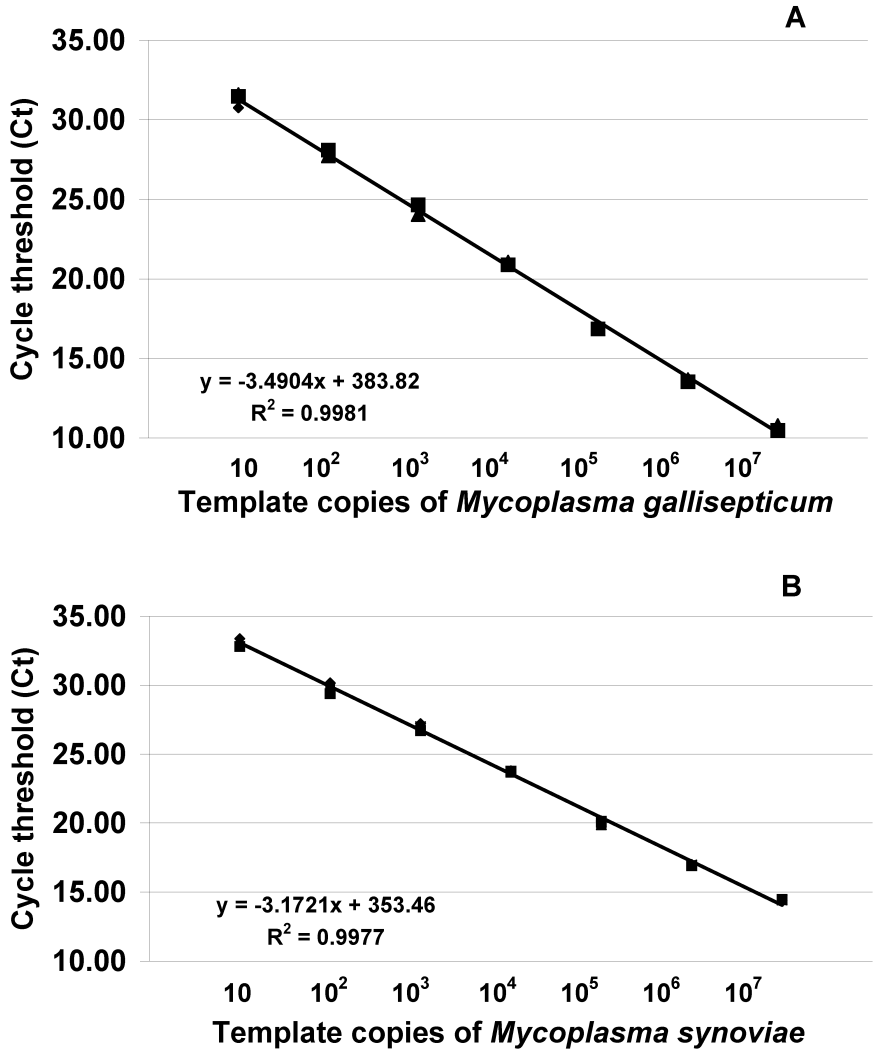


Figure 2. Real-time PCR amplification curve of MG and MS positive and negative controls and 6 positive samples with varying concentrations of template DNA. No positive control or positive sample was below the threshold of 400 fluorescing units.

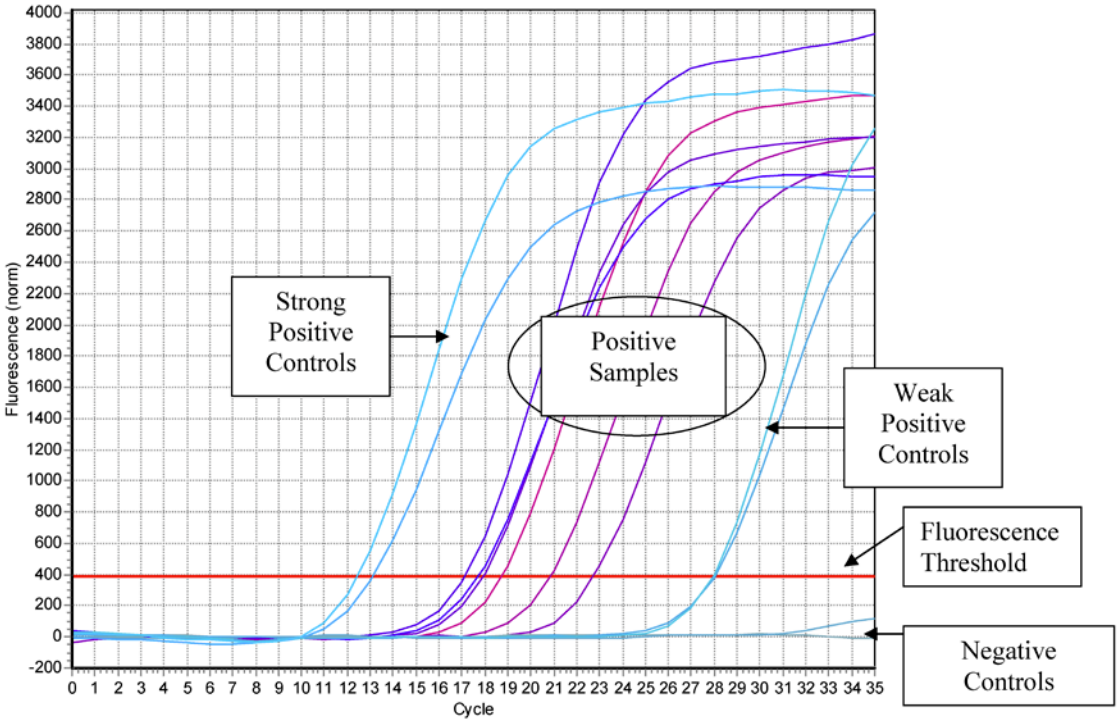
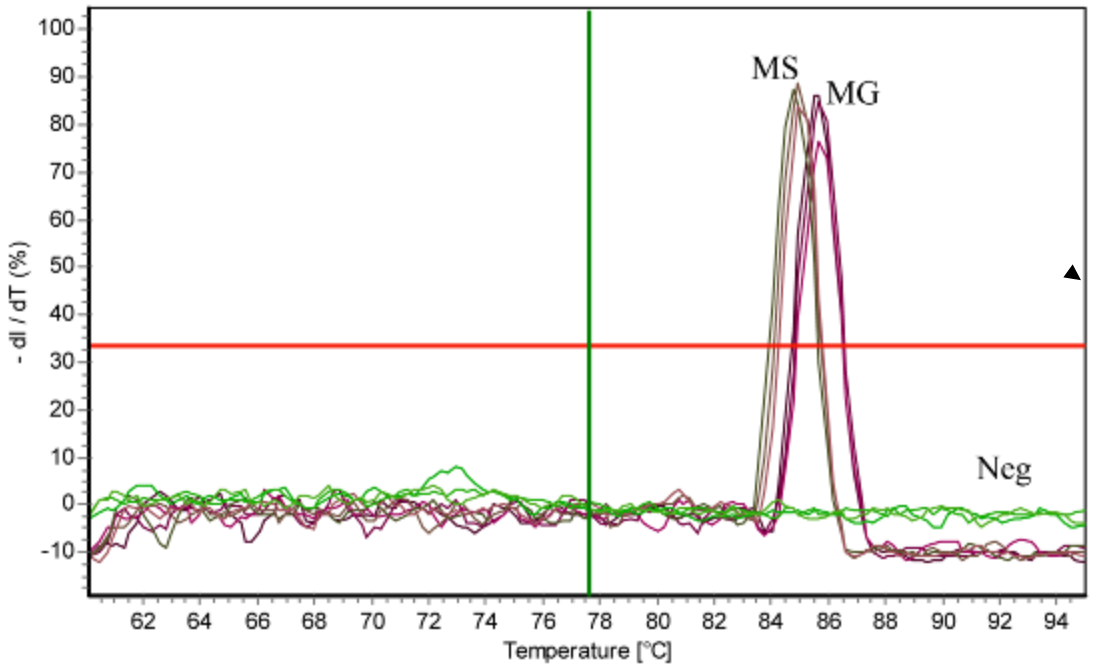


Figure 3. Real-time PCR melting curve analysis of the PCR amplicons for MG and MS positive and negative samples created from a10 fold-dilution series of prepared DNA. The melting temperatures of the amplicons (Tm) were calculated by the Eppendorf realplex software (version 2.0) to be approximately 85.7 and 85.0°C for MG and MS, respectively. Data is expressed as the negative first derivative of the melting curves as a function of temperature.



Conclusions

With an increase in consumption of animal derived foods, the number of broilers produced each year has increased. Furthermore, the number of foodborne illnesses associated with poultry has also increased in recent years. Improving production, reducing the economic losses due to poultry diseases and the food safety of poultry will be greatly improved by rapid and sensitive detection systems. Currently, no method of prevention is completely effective. Research and development of vaccines and other methods for proactive control is currently being performed. Thus, control through rapid diagnostics must be utilized to reduce the risk of loss of product and to improve the food safety of poultry products.

It was determined from these studies:

1) Optimized culturing of *Salmonella* isolated from poultry environments is performed by intial pre-enrichment in tetrathionate broth (TT) with subsequently enrichement in MSRV.

2) To detect live *Salmonella* cells in recently processed feed, targeting the *hilA* gene can improve sensitivity of the assay because *hilA* is upregulated due to heat stress encountered during processing and thus there is a relatively greater quantity of *hilA* target RNA for detection.

3) Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* can be performed simulataneously and in less than 1 hour utilizing a rela-time PCR assay which would facilitate control measures and reduce the economic loss of poultry production.